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Differentiation of Plasma Antithrombin Activities.* (20829)

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It has been suggested that the antithrombin activity of plasma can be divided into 4 effects, for which the names antithrombin I, II, III and IV were proposed(1). Antithrombin I refers to the adsorption of thrombin on fibrin. Antithrombin II is the plasma co-factor of heparin, which acts with heparin to interfere with the action of thrombin on fibrinogen. The term antithrombin III applies to the capacity of plasma to inactivate thrombin. This occurs independently of heparin. Antithrombin IV is the action of plasma which inactivates thrombin as it is being formed from prothrombin. This action does not destroy preformed, purified thrombin. This last antithrombin activity has been demonstrated only in ether treated plasma which is free of antithrombin III.

Although it has become possible to categorize plasma antithrombin activity into 4 distinct actions, it has not been established whether or not these effects represent four distinct chemical entities. In this paper information is presented which may offer some insight into this problem. It has also been of interest to consider the plasma factor which acts as a co-factor of heparin to inhibit the activation of prothrombin(2), and which is referred to as the '39 factor. This effect was discovered in 1939, and has not

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received further attention since that time.

Methods. In assaying for antithrombin II, 0.3 ml of bovine plasma or plasma fraction, which had been heated at 56°C for 3 minutes to remove fibrinogen, was added to 0.2 ml thrombin reaction mixture(3) and 0.1 ml of either saline or heparin (liquaemin-Roche-Organon, Inc., diluted 1/100 with saline). Then 0.3 ml of this mixture was added to 0.1 ml purified fibrinogen(4), and 0.1 ml purified thrombin(5) was added, and clotting times were noted. The concentration of the thrombin solution was 100 units/ml. A measure of the activity of antithrombin II could thus be obtained on the basis of the difference between the clotting times with and without heparin. Antithrombin III was assayed quantitatively by the method of Seegers, *et al.*(6). Antithrombin IV was determined on bovine plasma by the thrombin titer method described by Seegers, Johnson and Fell (1). In order to test fractions they were added to plasma so that the antithrombin IV activity could be compared with the plasma to which there was no addition. Before adding such fractions they were treated with $BaCO_3$ to remove prothrombin and with ether to remove antithrombin III. In assaying '39 factor, a prothrombin product(7) containing Ac-globulin was mixed with thromboplastin, calcium, and plasma or plasma fraction. Heparin, diluted 1/30 with saline, was also added. Thrombin concentrations were plotted against prothrombin activation time, and the '39 factor was estimated on the basis of the inhibition of thrombin formation with heparin, as compared with the formation of thrombin in an identical reaction mixture to which no heparin was added. Ether treatment for the removal of antithrombin III was carried out as previously described(1).

Results. The effects of various procedures on the antithrombin reactions are summarized in the table. Although an attempt was made to do exact quantitative studies, we have limited ourselves to qualitative statements, since the experimental error in some of our procedures may have been fairly large, and because quantitative standards for antithrombins II and IV, and the '39 factor have not as yet been established.

The three antithrombins and the '39 factor all resisted heating at 60°C for 3 minutes, but were largely or entirely inactivated by heating at 70°C. When plasma was dialysed against cold water to a specific resistance of 2500 ohms, all 4 factors were found in the water soluble fraction, but not with the water insoluble globulins. The factors are not adsorbed on $BaCO_3$. Crystalized bovine plasma albumin, a commercial product of Armour and Co., was assayed and found to contain none of the 4 factors.

It was reported previously that antithrombin III is removed from plasma or serum by treatment with ether, while antithrombin IV activity remains(1). This observation has been confirmed, and antithrombin II and '39 factor assays showed that these were also present in ether-treated plasma. It is of interest that antithrombin III activity could not be detected in the ether extract of plasma, or in a combination of such an extract with ether treated plasma. Therefore, while ether treatment is a valuable distinguishing feature, it does not necessarily establish a complete separation of antithrombin III from the other factors, since it is not thus far possible to decide whether antithrombin III is removed by ether, or simply modified and left in the plasma.

In the ammonium sulfate fractionations fresh bovine plasma was diluted with an equal volume of distilled water, fractionated at 50% and 70% of saturation, dialysed against water to a specific resistance of 1000 ohms, and lyophilized. The fractionation work was done at low temperatures. Samples from the fractions were then reconstituted to their original concentrations in physiologic saline.

The results of these experiments are summarized in the table. Antithrombin II was found in both the 0-50% and the 50-70% fractions, and in higher concentration in the latter fraction. Antithrombin III was found only in the 0-50% fraction, and in a concentration considerably lower than its concentration in unfractionated plasma. Antithrombin IV activity was not detected to any significant degree in any of the fractions, although there appeared to be a trace in the

TABLE I. Effects of Various Procedures on Antithrombins II, III and IV, and on the '39 Factor.

Procedures	Antithrombin II	Antithrombin III	Antithrombin IV	'39 factor
A. Heating plasma				
1. 60° for 3 min.	P*	P	P	P
2. 70° " 5 "	A	A	A	A
B. Dialysis of plasma against H_2O				
1. H_2O insol.	A	A	A	A
2. H_2O sol.	P	P	P	P
C. $BaCO_3$ treatment	N. ad.	N. ad.	N. ad.	N. ad.
D. Ether "	R	Re	R	R
E. $(NH_4)_2SO_4$ fractionation				
1. 0-50%	T	P	A	A
2. 50-70%	P	A	T	P
3. Supernatant from 70%	A	A	A	A

* P = Present; A = Absent; N. ad. = Not adsorbed; T = Trace; R = Remains; Re = Removed.

50-70% fraction. The '39 factor was found only in the 50-70% fraction. While our experiments did not demonstrate this factor in other fractions, this does not conclusively rule out the possibility that it was not present in these fractions, since other experiments indicated that a high concentration of accelerators of prothrombin conversion could overcome the inhibiting effect of heparin and its co-factor. We made a number of variations in our assays to take these variables into consideration.

Discussion. Previous work(6,8) has indicated that antithrombins II and III act in entirely different ways. The evidence indicates that antithrombin II acts with heparin to interfere with the action of thrombin on fibrinogen. Antithrombin III, on the other hand, probably interacts directly with thrombin so that the activity of thrombin is neutralized. In these interactions the activity of antithrombin III itself is neutralized. Heparin, in the concentrations used, has no effect on the antithrombin III. Ether treatment is also a means of differentiation, since antithrombin III activity is destroyed thereby, while antithrombin II is not. These considerations together with the distinction on the basis of ammonium sulfate fractionation indicate strongly that antithrombins II and III are two distinct chemical entities.

Although the evidence is far from conclusive, it seems quite possible that the '39 factor is identical with antithrombin II. This would then mean that heparin acts with the one co-factor at two distinct points in the blood coagulation mechanisms; namely at the prothrombin activation stage and at the fibrinogen activation stage.

It is interesting to note the report of Gorter and Nanninga(9) that heparin can act as an antithrombin in the absence of co-factor. They even consider the possibility that the co-factor does not exist. Perhaps their results may be understood on the basis of contamination of their crude thrombin preparation, or of their fibrinogen preparation, with co-factor, or perhaps simply on the basis of the very high concentration of heparin which they used. It is well established by their work, and also by that of Jaques(10), that heparin can interact with practically all proteins under suitable conditions. Therefore, heparin alone in high concentration might inactivate thrombin. In our own work heparin was quite dilute (one part in 10 of 1/100 heparin). In controls without added plasma or plasma fraction we did find a slight inhibiting action with heparin, of the order of about 3 seconds. However, on the addition of plasma, clotting times were prolonged for more than 50 seconds. At the same time, samples with and without plasma, in the absence of heparin, gave essentially the same clotting times. It seems clear therefore

that heparin requires a co-factor for full activity.

We are not yet able to relate antithrombin IV to the other antithrombins. It is possible that this activity represents another separate entity. It is also possible that more than one substance is involved, and that these substances are known by other manifestations in the complex interactions of the blood clotting mechanisms.

Summary. The antithrombin capacity of plasma can be differentiated into 4 main effects. Antithrombin I is the adsorption of thrombin on fibrin. Antithrombin II is the co-factor of heparin. Antithrombin III neutralizes thrombin activity even in the absence of heparin, while antithrombin IV destroys thrombin only while it is being formed from prothrombin. Some of the properties of antithrombins II, III and IV have been investigated, together with those of the factor which acts with heparin to inhibit the activation of prothrombin, and which is referred to as the '39 factor. The 4 activities have the common property of resistance to heating at 60°C for three minutes, but are destroyed by heating at 70°C. They are not adsorbed on $BaCO_3$. They are water soluble. None of these activities is found in a commercial crystalized bovine plasma albumin. Antithrombin III activity is the only one removed by ether treatment of plasma. In ammonium sulfate fractionation antithrombin II is found predominantly in the 50-70% fraction, while antithrombin III is found only in the 0-50% fraction. It is believed that these 2 factors are two distinct entities. The relation of antithrombin IV to these 2 is not yet clear. It is possible that the '39 factor and antithrombin II are identical.

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Monolayer Tissue Cultures I. Preparation and Standardization of Suspensions of Trypsin-Dispersed Monkey Kidney Cells.*† (20830)

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Recent reports by Dulbecco(1,2) on the use of monolayer tissue cultures for the study of certain animal viruses has made possible the application, in studies of animal viruses, of quantitative technics similar to those used with bacterial viruses. Dulbecco has demonstrated production of plaques of degenerated cells by Western equine encephalitis virus in monolayer chicken-fibroblast cultures(1) and similarly in monolayer monkey kidney cultures, plaques have been produced by the Brunhilde strain of Type 1 poliomyelitis virus(2).‡

Methods for treatment of kidney tissue fragments with trypsin to reduce the time required for cell outgrowth in plasma clot cul-

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‡ Dr. Renato Dulbecco very kindly made available full details of his report prior to its publication.

tures have been reported by Simms and Stillman(3) as well as by Robbins, Weller, and Enders(4). Dulbecco(2) has described a technic for dispersing monkey kidney cells with trypsin to obtain cell suspensions for preparation of monolayer cultures. It is the purpose of this report to describe certain modifications in the methods used by Dulbecco for preparing the trypsin-dispersed kidney cell suspension. These modifications include a) use of mechanical rather than manual agitation for dispersing the cells, b) use of a different nutrient medium, and c) quantitative adjustment of the dispersed cell suspension on the basis of total cell count or optical density. The procedures described are adaptable to the production of relatively large amounts of standardized dispersed cell suspension; the use of these suspensions for preparation of different types of monolayer cultures will be described.

Materials. All solutions used in this study were sterilized by Seitz filtration under positive pressure. *Phosphate buffered saline* (PBS), pH 7.5, was prepared according to the formula used by Dulbecco(2). *Trypsin solution* was prepared from Bacto-Trypsin 1:250 (Difco Corp., Detroit, Michigan) in a concentration of 0.25% by weight using PBS as diluent. The *horse serum* used in these studies was obtained from the blood of two horses and was heated at 56°C for 30 minutes before use. *Nutrient fluids—Synthetic mixture 199* of Morgan, Morton, and Parker (5) was used as the basic constituent of all media and differed from that described in the use of Hanks' balanced salt mixture in place of Earle's salt mixture. Since Hanks suggested separate addition of different quantities of sodium bicarbonate, this was used to permit adjustment of initial pH of the medium as desired for different purposes. Medium D, used for washing and suspending dispersed cells, contained the following ingredients per 100 ml of medium: synthetic mixture 199, 2.8% $NaHCO_3$, horse serum, 95, 3 and 2 ml, respectively. Penicillin and streptomycin were added to a concentration per ml of 100 units and 0.1 mg, respectively. The pH of this medium was 7.2-7.4 and upon incubation rose to 7.8-8.0.

Methods. Preparation of trypsin-dispersed cell suspensions. Kidneys were removed from healthy 5-7 lb rhesus or cynomolgus monkeys, exsanguinated under sodium pentobarbital anesthesia. The cortical area was dissected as free as possible from medulla, and minced with scissors into pieces about 4-5 mm in diameter. Minced cortical tissue was transferred to a 250 ml centrifuge tube, washed several times with PBS to remove red blood cells, and suspended in about 20 ml of trypsin solution per kidney. After 10 minutes at 37°C, the trypsin solution was discarded, replaced with about 20 ml of fresh trypsin solution per kidney and the suspension transferred to a Waring blendor the motor of which was controlled by means of a rheostat at a speed that produced agitation of the tissue without cutting or foaming. Mixing was allowed to proceed for 10 minutes. The supernatant fluid was saved after decanting through 3 layers of sterile cheesecloth to eliminate floating tissue particles and connective tissue fibers; the retained portion contained mainly clusters of cells, but also some single cells and cell debris. Fresh trypsin solution was added to the tissue remaining in the blendor and agitation repeated. After 8-10 such extractions, the tissue was near exhaustion as far as yielding additional cell clusters. Further extraction yielded increasing amounts of connective tissue fibers which were too fine to be retained by the cheesecloth filter and could not be separated easily from cellular components. The turbid trypsinized cell suspension was centrifugated at 1000 RPM for 5 minutes; the sediment was resuspended in approximately 20 volumes of medium D and centrifugated at 600 RPM for 2 minutes. Resuspension and washing was repeated 3 times at the slow speed. Final centrifugation of the pooled cell sediment was performed in graduated 15 ml centrifuge tubes. A yield of 1.0 to 1.5 ml of packed cellular material was usually obtained from the cortex of each kidney, the weight of which was approximately 4-5 g.

Standardization of cell suspensions. Packed cell sediment was diluted with medium D to make a 1:50 dilution based upon packed cell volume. An aliquot of this suspension was

further diluted to 1:200 for purposes of standardization. Since the work was done upon which this report is based, it has been found desirable to pass the 1:50 dilution through 3 layers of cheesecloth to remove aggregates of connective tissue fibers which appear to form as the result of packing of sediment during centrifugation. The *optical density* (O.D.) of the 1:200 cell suspension was determined in the following manner. Four ml of the cell suspension was acidified with 0.3 ml of 0.2 N HCl; acidification minimized variations in light absorption by phenol red in the medium. Optical density (neg. log of transmittance) was determined in a Coleman Junior Spectrophotometer using a wavelength of 590 mμ where light absorption by acidified phenol red was least. Four ml of similarly acidified medium was used as a blank. *Total cell count* was determined by enumeration of nuclei using an adaptation of the method suggested by Sanford and her associates(6). To 2.0 ml of the 1:200 cell suspension, in a graduated 15 ml centrifuge tube, was added 10 ml of 0.1 M citric acid. After one hour of incubation at 36°C, the tube was shaken vigorously and centrifugated at 1800 RPM for 15 minutes. The supernatant fluid was discarded and 1.0 ml of 0.1% crystal violet dissolved in 0.1 M citric acid added. The pellet was resuspended by mixing vigorously with a 1.0 ml pipette and the resulting suspension allowed to stand at room temperature for 5-10 minutes. At this time, 10 ml of 0.1 M citric acid was added, the tube thoroughly shaken and the contents centrifugated at 2000 RPM for 15 minutes. All but 1.0 ml of supernatant fluid was discarded, the volume brought to 2.0 ml with 0.5% Methocel§ (viscosity 4000 CPS) and the pellet containing nuclei resuspended in this solution. Nuclear counts were made without further dilution in a haemocytometer using the white blood cell counting squares; counts were expressed as the number of nuclei per ml of 1:200 cell suspension.‖

The relationship between O.D. and the nuclei count of the 1:200 dilutions of trypsin-dispersed cells, is illustrated in Fig. 1. Points are based on data derived from 16 successive batches of kidney, each utilizing tissue from 1 to 6 monkeys.

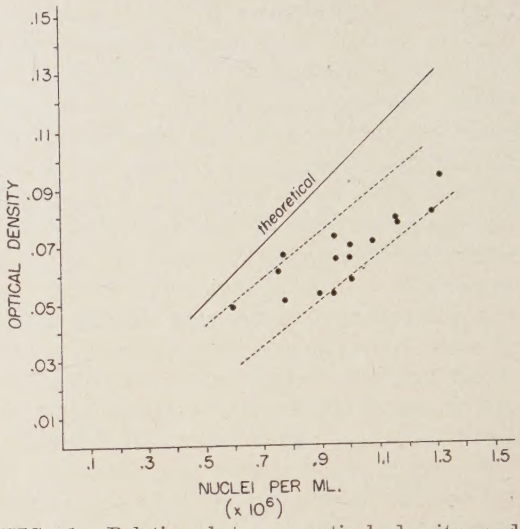

FIG. 1. Relation between optical density and nuclei/ml in 1:200 dilution of trypsin-dispersed cells.

On the assumption that there is a direct proportionality between O.D. and cell count, it is possible to envisage a straight line relationship if one were dealing with uniform suspensions of single cells. This theoretical relationship is illustrated graphically by the solid line in Fig. 1. Experimental points, plotted from data obtained with 16 preparations, all fall below the theoretical line. The most likely explanation for this observation is that cell suspensions are not composed of single cells but are made up of cell clusters of different sizes. The greater the number of cells in each cluster, the higher the total cell count in relation to the observed O.D. In addition, scattering of experimental points in Fig. 1 most likely is produced by variations in distribution of cell cluster sizes in different preparations. Data in Fig. 1 permit estimation of cell count by measuring the O.D.

§ A methylcellulose product which acts as a suspending agent, kindly furnished by Dow Chemical Company, Midland, Mich.

‖ The following formula was used to calculate number of nuclei/ml:

$$\frac{\text{Total nuclei in 4 white blood cell squares}}{4} \times 10000 = \text{nuclei/ml}$$

The 1:50 cell suspension stock is diluted with medium D to adjust to the desired number of cells per ml. Data in Fig. 1 indicate that 1:200 dilutions of trypsin-dispersed cells, adjusted on the basis of packed cell volume alone, contained between 600,000 and 1,320,000 cells per ml. It is possible to utilize the 1:200 dilution for preparation of cultures without additional standardization. However, standardization of cell content by the methods described permits utilization of more uniform cell populations as well as more economical use of cell suspension since it may contain sufficient cells to permit dilution to 1:300 or 1:400.

Maximum viability and reproducibility of results are obtained when dispersed cell suspensions contain 600,000 to 700,000 cells per ml. Such cell suspensions have been used to prepare several different kinds of cultures.

Preparation of cultures. Uniformity of suspension is maintained during distribution by the use of a magnetic stirring device together with a Cornwall automatic pipetting unit. *Petri dish (60 mm) cultures* are prepared with 4.0 ml of cell suspension containing 600,000-700,000 cells per ml, and incubated at 36°C in an atmosphere of 3% CO_2 as described by Dulbecco(2). Fluid is removed after 3 or 4 days of incubation and 4.0 ml of fresh medium D are added. After 6 or 7 days of incubation a confluent epithelial monolayer is developed.

To prepare *culture tubes*, 0.5 ml of suspension, containing approximately 300,000 cells, is added to screw-topped tubes (16 x 150 mm) with rubber inserts in the caps. These are incubated at a slight tilt from the horizontal at 36°C for 6 or 7 days without rolling; in this interval, a zone of confluent kidney epithelium covers the area of the tube in contact with cell suspension and pH of the medium has fallen to about 6.8. Between 800 and 1000 culture tubes can be prepared from cortical tissue of a single monkey by this method. Over 90% of tubes prepared in this manner are suitable for virus assay or other studies. For virus or antibody titrations medium D, containing horse serum, is not used. Instead, the medium employed is made up of 97.5 ml of mixture 199 to which is added 2.5 ml of 5% $NaHCO_3$. Cultures have also been prepared in *bottles* used normally for milk dilution tests. Each bottle receives 8.0 ml of standard cell suspension containing 600,000-700,000 cells per ml and is incubated without motion in a horizontal position for 6 or 7 days. After this interval, a confluent monolayer of kidney epithelium covers the entire flat glass surface (40 x 110 mm). Cultures can either be stoppered with rubber stoppers or with cotton plugs. In the latter case, incubation is carried out in an atmosphere of 3% CO_2. The application of this type of culture for the propagation of poliomyelitis viruses will be reported separately.

Viability of cell suspensions is retained at a usable level for at least 2 days beyond the day of preparation when kept at 4°C. For convenience, suspensions are stored as 1:50 dilutions and brought to the desired concentration with fresh medium D just before use.

Summary. An adaptation of the method of Dulbecco for preparation of trypsin-dispersed monkey kidney cell suspensions has been described. Procedures employed yield quantitatively standardized cell suspensions which can be used in the preparation of large numbers of replicate cultures.

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Ascorbic Acid and Ethylene Diamine Tetraacetate as Antidotes in Experimental Vanadium Poisoning. (20831)

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It has been recently shown that the calcium disodium salt of ethylenediamine tetra-acetic acid ($CaNa_2EDTA$) affords protection against lethal doses of vanadium in mice(1). In a study of vanadium toxicity by Proescher *et al.*(2), it was suggested that the mode of detoxication of vanadium might be reduction in the body, from the evidence that the lower valence states of vanadium appeared to be less toxic than the higher valence states. It was therefore thought possible that ascorbic acid, a reducing substance, might also decrease the toxicity of vanadium. Accordingly, comparative tests of antidotal effectiveness of ascorbic acid and $CaNa_2EDTA$ were made in 3 animal species and the probable detoxication mechanism of the former antidote has been elucidated by polarography.

Methods. Mice of 20-29 g body weight of the Webster† strain and rats, 100-150 g body weight of a Wistar-Carworth† derived strain were used. Dogs were male mongrels, 6-12 kg in weight. The form of vanadium for administration was $NaVO_3 \cdot H_2O$‡ in isotonic saline solution. As used for mice, the concentration was 4 mg/ml (pH 7); for rats, 1% solutions (pH 7.85), and 3% solutions (pH 7)§ for the dogs. Calcium disodium ethylenediamine tetraacetate was chosen as the EDTA salt because of the reduced tendency of this salt to combine with the blood calcium(3). Ascorbic acid solutions (ca. pH 2.5) prepared without neutralization seemed to be well tolerated. Mice were injected intraperitoneally with ascorbic acid, 20 minutes prior to vanadium administration by the same route. Rats were injected with vanadium subcutaneously and were given the antidotes intraperitoneally 5 minutes later when signs of toxicity had become manifest. An observation period of 7 days was used in all mouse and rat tests. Dogs were injected intramuscularly with vanadium and the antidotes administered intraperitoneally after the first vomiting episode. Polarographic studies were made with a visible recording Sargent Model XXI instrument. Phosphate buffer, pH 7.4, gave no evidence of significant complex formation with vanadium. Each determination was made at 30°C on a 20-ml aliquot containing 0.01% gelatin as a maxima suppressor.

TABLE I. Protection of Mice by Ascorbic Acid against the Toxicity of Vanadium. Mortality ratios expressed as number of animals that died (numerator) to those used (denominator).

mg/kg $NaVO_3 \cdot H_2O$	mg/kg as V	Controls, no ascorbic acid	Ascorbic acid (1 g/kg), 20 min. before vanadium inj.
30	10.9	14/20	1/20
40	14.6	19/20	0/20

Results. Mice. Table I shows the protection afforded mice by ascorbic acid, intraperitoneally administered in a dose of 1 g/kg, 20 minutes prior to vanadium administration. Protection was essentially complete at doses corresponding to the LD_{70} and LD_{95} levels of $NaVO_3 \cdot H_2O$ (30 and 40 mg/kg, respectively). Ascorbic acid injected alone produced no signs of toxicity in 20 mice other than slight transitory depression. In subsequent tests, doses as low as 125 mg ascorbic acid/kg body weight afforded essen-

TABLE II. Antidotal Action of $CaNa_2EDTA$ and Ascorbic Acid in Vanadium-Poisoned Rats. Antidotes administered 5 min. after vanadium. Mortality ratios expressed as number of animals that died (numerator) to those used (denominator).

mg/kg $NaVO_3 \cdot H_2O$	mg/kg as V	Controls	$CaNa_2$-EDTA, 1 g/kg	Ascorbic acid, 250 mg/kg
60	21.9	20/20	7/20	6/20

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† Obtained from Hamilton Laboratory Animals, Inc., Cincinnati, O.

‡ Obtained from Fisher, Eimer and Amend.

§ The more concentrated 3% metavanadate solutions required neutralization with HCl to bring to a more physiologic pH value.

tially complete protection against an LD_{70} of $NaVO_3 \cdot H_2O$.

Rats. Table II shows the dosage-mortality data for rats when either $CaNa_2EDTA$ or ascorbic acid was administered after vanadium had given rise to manifest signs of toxicity. The 60 mg/kg dose of $NaVO_3 \cdot H_2O$ was MLD_{100} by subcutaneous administration. When $CaNa_2EDTA$ (1 g/kg) was administered 5 minutes after the vanadium, 65% of the rats survived. Ascorbic acid (250 mg/kg) administered in the same manner resulted in 70% survival.

Dogs. The intramuscular MLD of 10 mg $NaVO_3 \cdot H_2O$/kg was found to produce death within 18-36 hours with survival in only an occasional animal after a 7-day semicomatose state. Using this dose, 3 dogs were given $CaNa_2EDTA$ (100 mg/kg) intraperitoneally, after the first vomiting episode had occurred. Two more doses of EDTA at the same level and by the same route were administered at 2 and 4 hours after the first injection. Twenty-four hours later the dogs appeared to be in fair condition, and within 48 hours, the dogs were eating and showed few or no signs of vanadium toxicity. Similar responses were obtained after 3 more dogs were given 10 mg $NaVO_3 \cdot H_2O$/kg, followed by 50 mg ascorbic acid/kg‖ after the first toxic signs. Two subsequent injections of ascorbic acid were given, of the same dose and by the same route, at 2 and 4 hours after the initial injection. The ascorbic acid-treated dogs appeared to recover more quickly and showed fewer signs of toxicity than the EDTA-treated dogs. The dogs that had been treated with the antidotes appeared to be in good health and remained so for an observation period of 8 weeks after the V injection.

Polarographic studies. Ascorbic acid produced an anodic wave at an observed half-wave potential ($E_{1/2}$) of –0.01 volt. Current-voltage at different V^{+5} concentrations produced ill-defined, double waves measurable quantitatively within ±20%. A potential of –0.17 volt was taken as a reproducible $E_{1/2}$ for V^{+5} at pH 7.4 and was used as a reference for subsequent work. Similar findings have been previously described for V^{+5} in oxalate solutions(4). To determine the effects of ascorbic acid on V^{+5}, a polarogram was made of a solution containing 77 μg $NaVO_3 \cdot H_2O$/ml of phosphate buffer at pH 7.4; subsequently ascorbic acid, sufficient to give a concentration of 385 μg/ml, was added to the oxygen-free solution. Polarograms of this solution made at intervals of 15 minutes indicated that after the first interval approximately 50% of the V^{+5} had disappeared, and after one hour, 100%. This solution, buffered with acetate to pH 5.6 gave a double anodic wave characteristic of V^{+2}, thus suggesting that V^{+5} is reduced by ascorbic acid to V^{+2}. Hydrogen peroxide, an associated oxidation product of ascorbic acid, was believed not to be the agent involved in this reduction because the polarographic waves did not simulate those developed from the addition of ascorbic acid to V^{+5} solution. After reduction of V^{+5} with ascorbic acid bubbling air through the solution for several hours oxidized all the ascorbic acid, and the V returned to the pentavalent state as evidenced by the reappearance of the characteristic V^{+5} curve.

To observe the effect of an acid without strong reducing powers, gluconic acid was added to a fresh solution of $NaVO_3 \cdot H_2O$. The resulting curve resembled the V^{+5} curve, but was greatly expanded, and the observed $E_{1/2}$ was shifted slightly, thus suggesting the formation of a vanadium complex(5). The failure of V^{+5} to disappear in this experiment showed that gluconic acid does not reduce vanadium as does ascorbic acid.

$CaNa_2EDTA$ added to a solution of $NaVO_3 \cdot H_2O$ did not change the wave pattern, but shifted the $E_{1/2}$ slightly. ($CaNa_2EDTA$ concentration, 1540 μg/ml; $NaVO_3 \cdot H_2O$ concentration, 77 μg/ml.) The observed response suggests a very loosely-bound complex of vanadium with the chelating agent. The $E_{1/2}$ of a vanadyl sulfate (V^{+4}) solution was shifted slightly when treated with $CaNa_2EDTA$ indicating the probable formation of a weak complex similar to that found with V^{+5}.

Discussion. In mice and rats, signs of

‖ This dose of ascorbic acid for dogs was approximately 5 times that of the metavanadate on a mg basis; that for rats (250 mg/kg) and mice (125 mg/kg) approximately 4 times.

vanadium toxicity were usually present for 2 hours or more after $CaNa_2EDTA$ was administered to vanadium-poisoned animals; however, recovery gradually occurred in the majority of the animals. Dogs behaved similarly, with signs of vanadium toxicity being present for some time after $CaNa_2EDTA$ administration, with gradual, but eventual, recovery.

When ascorbic acid was administered to vanadium-poisoned animals, the signs of vanadium toxicity appeared to be arrested within one-half hour and recovery appeared to be more rapid than with $CaNa_2EDTA$. It has been reported(6) as well as indicated from the present studies, that EDTA forms a loose complex with vanadium which might explain why a short period of time is required before the signs of toxicity begin to subside in vanadium-poisoned animals, after EDTA administration. On the other hand, ascorbic acid was found to react rapidly with $NaVO_3 \cdot H_2O$ *in vitro* from polarographic studies, in which approximately 50% of the pentavalent vanadium disappeared from solution within 15 minutes after addition of ascorbic acid. Unfortunately it was not possible to decide from these determinations alone whether ascorbic acid formed a complex with the vanadium it reduced. Only 50% ascorbic acid was recoverable, however, by actual analysis from an equimolar mixture of vanadium and ascorbic acid shortly after mixing. A reasonable assumption is that the remainder was oxidized to noncomplexable moieties.

It has been shown(7) that acidity decreases the toxicity of injected vanadium; however, this is not believed to be the chief mode of action of ascorbic acid in these antidotal tests. Relatively large doses of ammonium chloride were required to give only a moderate prophylactic protection, whereas far smaller doses of ascorbic acid afforded high protection against vanadium. Moreover, in the smaller effective doses employed, 125 mg/kg, ascorbic acid would not be expected to alter appreciably the tissue milieu.

Summary. 1. Ascorbic acid at 1 g/kg when administered prior to vanadium antagonizes the toxicity of vanadium as $NaVO_3 \cdot H_2O$ in mice at the LD_{70} and LD_{95} levels. Dosage levels as low as 125 mg/kg ascorbic acid showed a protective action against the LD_{70} of $NaVO_3 \cdot H_2O$ in mice. 2. Antidotal effects of calcium disodium ethylenediamine tetraacetate and ascorbic acid have been demonstrated in vanadium-poisoned rats and dogs, after signs of toxicity had become manifest. 3. Ascorbic acid is probably the more rapidly-acting of the 2 antidotes. Signs of vanadium toxicity were present for a longer period of time in vanadium-poisoned animals after $CaNa_2EDTA$ than after ascorbic acid administration. 4. One possible mode of detoxication of vanadium by ascorbic acid is suggested, on the basis of *in vitro* polarographic studies, to be reduction of the vanadium.

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Effect of Malonate on Bacteremia Developed in Mice Artificially Infected with *Salmonella typhimurium*.*† (20832)

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Survival time of mice artificially infected by intraperitoneal injection of a standardized suspension of *Salmonella typhimurium* is reduced from an average of more than 72 hours to less than 24 hours by injections of sublethal quantities of certain inhibitors and intermediates of the tricarboxylic acid cycle (1-3). Malonate is the most effective compound (10 have been tested but results with only 5 have been reported) in reducing survival time in infections not only with this particular organism but also with *Proteus morganii, Micrococcus pyogenes var. aureus,* and *Streptococcus pyogenes*(4). Implied in these observations is a degree of specificity between the particular type of alteration in the tricarboxylic acid cycle of the host and the ability of the bacterial parasite to "exploit" it.

Some years ago, Webster(5) cultured at intervals the blood of mice infected intraperitoneally with *S. enteritidis*. Positive cultures were obtained after 2 to 4 hours but they became negative at about 12 hours. They remained negative until the onset of the terminal stages of the disease. Since Webster's procedure was not designed to yield quantitative data, a modified dilution count procedure was devised which permits repeated bacterial counts on the blood of individual mice.

Methods. Ten mice were infected intraperitoneally with 0.5 ml of a saline suspension containing about 250,000 cells of *S. typhimurium*. Starting immediately thereafter and continuing at hourly intervals for a total of 8 injections, half the mice were injected with 20 mg sodium malonate (Eastman) contained in 0.5 ml of sterile saline and the remaining half, to serve as controls, were injected concomitantly with the same volume of sterile saline. Each mouse was marked by color code in such a way that it could be readily identified. As soon as the second injections of malonate or saline had been completed, a blood sample was taken, as described below, from each animal in turn and when all 10 mice had been bled, they were sampled again in the same order, over and over for a period of about 12 hours. Each time an animal was bled, the end of the tail was amputated and blood was drawn to the "1.0" mark in a small red blood cell diluting pipette. The absolute volume of this portion of the pipette was determined in advance by filling it to the mark with mercury and then weighing this amount of mercury on an analytical balance. Only pipettes with a volume of 1.0 cu mm $\pm$ 5% were used. The blood was diluted to the "101" mark of the pipette with sterile heparinized saline. For each dilution a different tube of saline was employed. The pipette was thoroughly shaken and its contents were then blown onto the surface of SS agar (Difco) contained in a Petri dish. The diluted blood

TABLE I. Number of Mice Dying from Intraperitoneal Infection with *S. typhimurium* followed Immediately by Hourly Injections of Either 20 mg Sodium Malonate Contained in 0.5 ml of Sterile Saline for a Total of 8 Injections or a Comparable Number of Injections of 0.5 ml of Saline. (15 mice per group.)

Time post-infection (hr)	Death of mice injected with Malonate	+ Saline
7	0	0
7½	1	0
8	4	0
9	5	0
10	6	0
13	10	0
24	15	0
72	15	2
96	15	4
120	15	13
144	15	15

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was spread over the agar with a wire loop, the plate was incubated over night at 37°C and the number of colonies times 1,000 (since 0.001 ml of blood was used) was taken as the number of bacteria per ml of blood. When preliminary counts indicated that the number of bacteria was likely to approximate 200,000 or more per ml of blood, a second dilution was necessary. This was accomplished by discharging the contents of the red cell pipette onto a clean glass slide and then drawing it up into a white blood cell diluting pipette (similarly calibrated) to the "1.0" mark. The white cell pipette was then filled to the "11" mark with saline, the contents were shaken and discharged onto SS agar. The white cell pipettes were selected with capillary volumes of 9.0 $mm^3 \pm 5\%$. Therefore, the number of colonies developing on the SS agar times 11,000 gave the estimated number of bacteria per ml of blood. The factor of 11,000 is obtained since 9/101 mm^3, or about 1/11,000, ml of blood was employed.

Results. As a check on the method, quadruplicate counts were made by both the single and the double dilution technics on appropriate dilutions of a broth culture of *S. typhimurium*. The culture used for the latter count was diluted 1:10 for the former. The average count obtained by the single dilution was 26,300 with a range of 21,000 to 29,000. The average count for the double dilution was 255,000 with a range of 231,000 to 297,000. These results are adequate for the purposes of the present experiments.

The procedure, outlined above, was repeated three times or until a total of 15 mice in each group was studied. A summary of the results is shown in Fig. 1. The logarithm of the average number of bacteria found in the blood of mice injected with malonate and of mice injected with saline is each plotted against time post-infection. The curve designated as "malonate-treated" rises steadily to a peak value in excess of 3 million bacteria per ml of blood at 9 hours and this high count is maintained for the next 3 hours. The mortality data for the same mice are given in Table I, column 2. Ten of the 15 mice died between 7½ hours and 13 hours post-infection while the remaining 5 were dead at 24 hours,

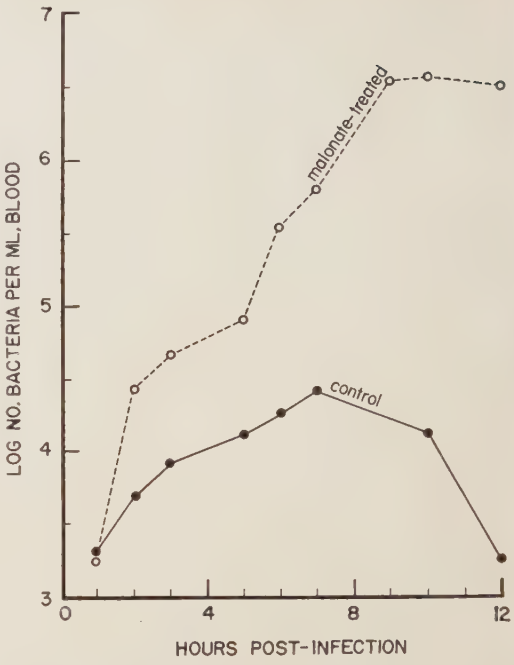

FIG. 1. Logarithm of mean number of bacteria/ml of blood plotted against hours post-infection for 15 mice given hourly injections of 20 mg sodium malonate contained in 0.5 ml of saline for a total of 8 injections (malonate-treated) and for 15 mice given the same number of injections of 0.5 ml saline (controls). All mice were infected intraperitoneally with about 250000 cells of *S. typhimurium* contained in 0.5 ml of a saline suspension.

the exact time of death not having been determined. The constancy of the bacteremia in these mice between 9 and 12 hours post-infection must be considered characteristic of the surviving animals and not necessarily evidence for a static bacterial population. Control mice show a peak bacterial count at 7 hours. By the twelfth hour post-infection the bacteria have practically disappeared from the blood stream (it was zero for 7 of 10 mice on which counts were made). This agrees, in general, with Webster's findings(5). As Table I, column 3 shows, the control mice died between 72 and 144 hours. The average bacterial count for 5 control mice at 72 hours was 406,000. Thus the bacteremia becomes more severe as the infection progresses.

Conclusions. These experiments show that malonate injections in mice artificially infected with *S. typhimurium* results in the development of a bacteremia more than 100 times as

severe as that found within the same time period in control mice injected with equal volumes of saline. Malonate injections in the same mice also reduce survival time, apparently because of the fulminating infection which results. The increased severity of the bacteremia accompanying the malonate injections could be due to a) an impairment of the mouse's defense against bacterial infection, b) the establishment of an internal environment within the mouse which favors bacterial reproduction along the lines postulated by Lewis(6), or c) a combination of (a) and (b). The need for additional experiments thus becomes evident.

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Effect of Glycol Mesylates on Mouse Sarcoma 180.* (20833)

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Haddow and Timmis(1) recently reported on the inhibition of growth of Walker carcinoma 256 in rats by a series of α,ω-dimethanesulfonyloxyalkanes, $CH_3SO_2O(CH_2)_nOSO_2CH_3$. Growth inhibiting activity was found to be greatest when $n = 4$ or 5. Galton (2) tested Myleran, the compound corresponding to $n = 4$, and found it to be of value in the treatment of chronic myeloid leukemia. We are reporting on the ability of a series of methanesulfonyl ("mesyl") esters of primary, secondary and tertiary aliphatic diols and triols, and the mesylates of polyglycols, to inhibit the growth of Crocker sarcoma 180 in white mice.

Materials and methods. Both Carworth Farms and Taconic white mice were employed. Drugs were injected intraperitoneally in normal saline or peanut oil both in the determination of single-dose LD_{50} values and in the evaluation of tumor inhibitory activity. A 7- to 10-day-old tumor was dissected in penicillin-Ringer-Locke solution, and approximately 20 mg portions transplanted subcutaneously into the axillary region of anesthetized mice, groups of 5 experimental and 5 control animals being used with each drug at each dose level. Treatment was begun 24 hours after transplantation, and was continued daily or on alternate days dependent on the reaction of the mice to the drug. Initially the compounds were administered at a level $\frac{1}{3}$ to $\frac{1}{2}$ that of the single-dose LD_{50} value, the level subsequently being reduced if toxic effects became marked. The experiments usually were terminated eight days after transplantation, and the mean weights of the control and treated tumors determined. The compounds were compared on the basis of the mean per cent change in body weight in the experimental and control animals, and the mean per cent inhibition of tumor weight. As a control, mice were treated with 2:4:6-tris-ethyleneimino-1:3:5 triazine (TEM), a known inhibitor of sarcoma 180. In the case of several of the more active compounds, hematoxylin and eosin stained slides were prepared from tumors recovered from treated animals, and compared with tumors from control animals.

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† From a thesis by Charles C. Morgan to the Graduate School of the University of Alabama in partial fulfillment of the requirements for the degree Master of Science.

TABLE I. Inhibition of Growth of Sarcoma 180 in Mice by Methanesulfonyl Esters of Aliphatic Diols, Triols and Polyglycols.

Hydroxy compounds from which mesyl esters were derived	LD_{50}, mg/kg	Treatment level, mg/kg	Mean % change in body wt		Mean % inhibition of tumor wt
			Treated mice	Control mice	
Diols		CH_3SO_2-O-$(CR_2)_n$-O-SO_2CH_3 (R=H or alkyl groups)			
n=2					
1 Ethylene glycol	250	85	— 3.0	+ 3.0	26
2 Propylene "	500	170	— 7.8	— 5.3	4
3 Butanediol-2,3	800	235	— 3.8	— 5.1	41
4 Pinacol	30	17*	— 1.3	— 3.1	15
n=3					
5 Trimethylene glycol	500	235	— 8.2	+ 2.2	37
6 Butanediol-1,3	500	150	— 2.2	+ 7.4	53
7 2,2-Diethyl propanediol-1,3	1800	800*	+ 5.1	+10.8	0
8 2-Butyl-2-ethylpropanediol-1,3	500	100*	+10.5	+20.2	0
9 2-Methyl-pentanediol-2,4	1000	500	—23.0	+ 9.5	79
10 2-Ethyl-hexanediol-1,3	800	400	— 7.4	— 3.2	40
n=4					
11 Butanediol-1,4	250	40*	—19.9	+ 3.7	92
12 2-Butynediol-1,4	15	5*	— 4.6	— .5	0
13 2,5-Dimethyl-3-hexyne-diol-2,5	200	80*	— 6.4	— 3.0	0
14 3,6-Dimethyl-4-octynediol-3,6	1200	333*	— 6.6	— 3.1	26
n=5					
15 Pentanediol-1,5	2000	500	— 9.8	+ 5.9	42
16 2-Methoxymethyl-2,4-dimethyl-pentanediol-1,5	300	100*	— 2.8	— 1.2	31
Triols					
17 Hexanetriol-1,2,6	1500	300*	—11.0	+ 6.2	89
18 Triol 230†	1200	400*	— .5	+ 5.0	35
Polyglycols		CH_3-SO_2-O-$(CH_2CH_2$-O$)_n$-CH_2CH_2-O-SO_2CH_3			
19 Diethylene glycol	500	165*	+ 2.0	+ 6.0	62
20 Dipropylene "	1000	300*	—31.2	+ 1.0	95
		150*	—25.9	+ 1.6	64
n=2					
21 Triethylene glycol	1500	700	—12.6	— .6	61
		1000	—25.0	+10.2	78
n=21					
22 Polyglycol E-1000	2000	666	+ 1.2	+ 7.7	14
n=44					
23 *idem* -2000	5000	2000	—11.7	— 9.5	15
n=90					
24 *idem* -4000	10000	3000	— 5.9	— 6.4	0
n=135					
25 *idem* -6000	10000	5000	— .5	+ 2.0	38
n=200					
26 *idem* -9000	10000	1000*	— 3.3	+ 9.2	57
		5000*	+ 4.1	+ 9.0	46
27 TEM	2.8(3)	.5*	—21.1	+ 1.6	90
— Starvation‡	—	—	—28.3	+ 6.9	30

* Treatment on alternate days. † 90% 2-hydroxyethoxymethyl-2,4-dimethyl-pentanediol-1,5.
‡ See text.

Results. The results obtained in screening this series of methanesulfonyl esters of glycols and polyglycols are presented in Table I. Among the alkylene diols, the activity reported by Haddow and Timmis(1) for the mesylate of butanediol-1,4 was confirmed. The introduction of unsaturation into the carbon chain reduced tumor inhibitory ac-

tivity, usually with an increase in toxicity. Variations in the degree of branching in the carbon chain, or in the degree of substitution on the carbon atoms bearing OH groups, did not have a uniform effect on either toxicity or tumor inhibition. Of the 2 triols investigated, the trimesylate of hexanetriol-1,2,6 was especially effective. The mesylates of the polyglycols were studied to determine the effect of the introduction of oxygen into the carbon chain, and because of the increased water solubility of these compounds. As a group, these compounds exhibited a low order of toxicity, but significant inhibition of tumor growth was observed only when they were administered at levels causing definite reduction in body weight.

Because compounds are known which inhibit tumor development mainly as a result of the inanition resulting from administration of the drugs, the effect of starvation on the growth of sarcoma 180 in mice was determined. During an 8-day experimental period, mice were permitted free access to water but food was withheld except for one Purina pellet per animal on the fourth day. Such caloric restriction was found to result in a mean loss in body weight of 28.3%, accompanied by a 30% inhibition in tumor weight. The reduction in tumor weight caused by compounds such as number 9, 11, 17 and 20 thus is considerably in excess of that caused by caloric restriction alone.

The dimesylate of triethylene glycol (No. 21) caused a toxic reaction not observed with the other drugs in this series. Following injection of this compound the animals became convulsant within 5 to 10 minutes with full extension of all extremities and with the body in an opisthotonic position. Sections for microscopic study were prepared from 7-day control tumors, and 7-day tumors from animals treated with compounds 11, 17, 20 and 21. In comparison with the control tumors, the treated tumors were characterized by a scarcity of mitotic figures and an increase in fat and intercellular tissue.

Summary. Methanesulfonyl esters of a series of aliphatic diols, triols and polyglycols were prepared and tested for an ability to inhibit the growth of sarcoma 180 in white mice. Variations in the degree of branching in the carbon chain, or in the degree of substitution at the carbon atoms bearing hydroxyl groups, did not have a uniform effect on either toxicity or tumor inhibitory activity. Significant inhibition of tumor growth could be demonstrated for 5 of the drugs when they were administered at levels causing definite reduction in body weight.

We wish to thank the following companies for samples of compounds used in the synthesis of the methanesulfonyl esters: General Aniline and Film Corp., 2-butyne-1,4-diol; E. I. du Pont de Nemours and Co., pentanediol-1, 5; Carbide and Carbon Chemicals Corp., 2,2-diethyl propanediol-1, 3; 2-butyl, 2-ethyl propanediol-1,3; butanediol-1,3; 2,3-butylene glycol; 2-methyl-2,4-pantanediol-2,4; 2-methoxymethyl-2,4-dimethyl pentanediol-2,4; triethylene glycol; 2-ethyl hexanediol-1,3; Triol 230; dipropylene glycol; hexanetriol-1,2,6; The Dow Chemical Co., polyglycols E-1000 to E-9000.

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Influence of Cortisone upon Brown Fat of Hamsters and Mice.* (20834)

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In the course of studies on the extraneural progression of parenterally inoculated poliomyelitis virus (strain MEF_1) into cortisone-treated Syrian hamsters, a selective affinity for brown (hibernating) fat was noted. White fat was not involved. The differential lipotropism was exemplified by microscopic lesions confined exclusively to brown fat even in areas where the two forms of adipose tissue were in intimate contact. Viral assay further confirmed the selectivity of involvement(1-4). To further the understanding of the role played by brown fat in experimental poliomyelitis, an investigation was undertaken to evaluate the action of cortisone upon brown fat of hamsters and mice.

Experimental. In the first series of experiments, 200 untreated male hamsters were sacrificed at varying ages in order to establish normal growth curves for the two types of lipid-containing tissues. Preliminary dissections demonstrated that the mass of brown fat, situated between the mesial aspects of the scapulae, remained a constant fraction of the total mass of body brown fat, irrespective of age. The interscapular brown fat body (ISB) is a discrete, encapsulated, bilobate structure which is superficially located and readily resected with accuracy. The demarcated inguinal fat pad in the hamster is composed almost exclusively of white fat. These two fat masses were dissected out and their weights used as the representation of total body brown fat and white fat respectively. A number of ISB, thus removed from normal animals of different ages were examined histologically for the presence of infiltrating white fat cells. Counts were made repeatedly in order to determine the ratio of the two varieties of lipid cells. The second series of experiments was designed to investigate the effect of cortisone upon the weight and histological appearance of brown and white fat in hamsters and mice. Forty prepubescent, male hamsters between 25-30 g were each injected intramuscularly with 12 mg of cortisone (3 mg, 4 times, given over a period of 2 days), and groups of 10 animals were killed at 2, 4, 7 and 10 days after commencement of cortisone administration. Forty uninjected prepubescent male hamsters, serving as controls, were sacrificed at the same times. An additional 54 male hamsters of widely varying ages, weighing from 12-80 g, were treated with cortisone in total doses ranging from 4-24 mg, and sacrificed 7 days later. Forty male Swiss mice weighing between 14-17 g received 9 mg of cortisone intramuscularly (3 mg, 3 times, over a period of two days), and were sacrificed in groups of 10, at the time intervals stated above. Forty untreated mice served as controls. The interscapular brown fat body and the inguinal white fat pad were dissected out in all animals, weighed on a Roller-Smith balance, and equated to mg/100 g body weight.

Growth pattern of brown and white fat in the normal hamster. Up to a certain age the inguinal fat pad (white fat) increases proportionately to the weight of the body, *i.e.*, about 250 mg/100 g body weight in the 25 g male hamster and about 1,250 mg/100 g body weight in the 75 g animal. Beyond this stage, the proportion of inguinal fat to body weight maintains a relatively stationary ratio. The *interscapular brown fat body* rises sharply in weight to a level of approximately 260 mg/100 g body weight in the 20 g hamster. Beyond this body weight there is no further increase in the relative weight of the brown fat body. In the 75 g hamster, this structure weighs about the same, *i.e.*, 260 mg/100 g body weight (Graph 1). In hamsters weighing 20 g or less, the interscapular brown fat body is composed exclusively of brown fat cells, as determined by histologic sections. These cells are smaller in diameter than adult

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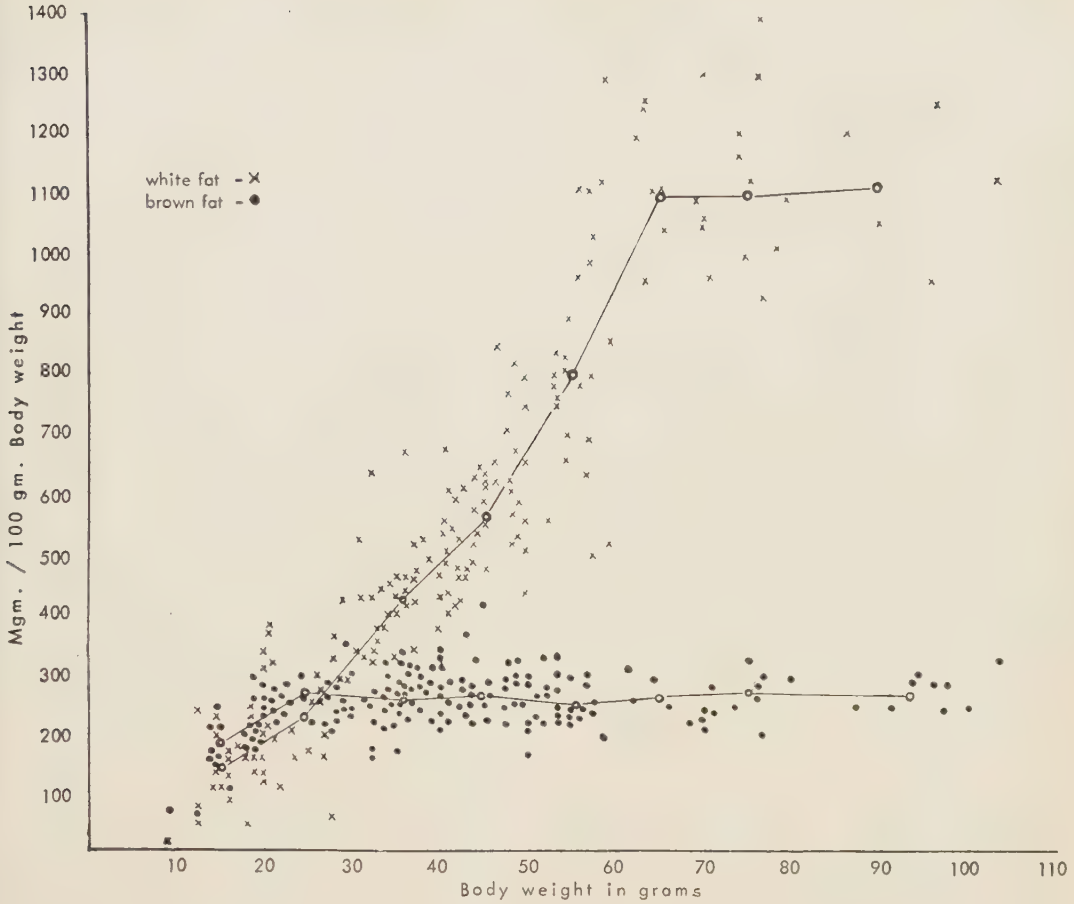
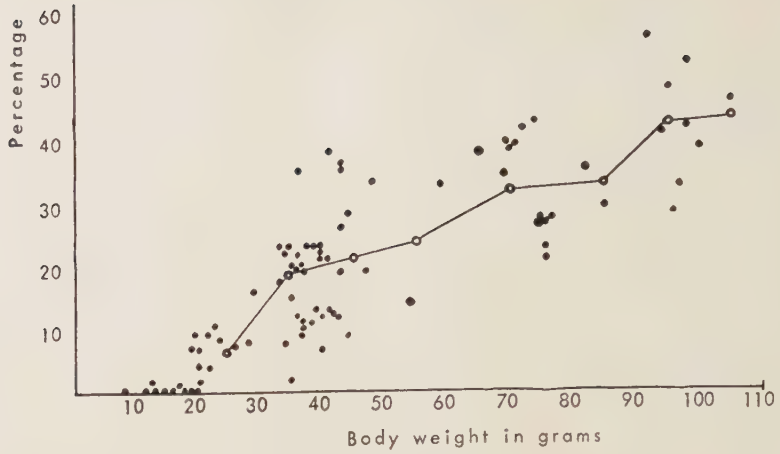


GRAPH 1. Growth curves of brown fat (ISB) and white fat (inguinal fat pad) in the normal male Syrian hamster.

fat cells and are characterized by polygonal contour, centrally situated nuclei and polylocular cytoplasmic vacuolization. With progressive increase in body weight, a greater number of typical white fat cells appears within the confines of the ISB capsule. The emergent white fat cells are generally restricted to the peripheral subcapsular region. In the 80 g hamster, white fat elements constitute about 50% of the ISB cell population (Graph 2).

Effect of cortisone upon brown and white fat of hamsters and mice. On the second day after cortisone inoculation into the prepubescent male hamster, a moderate increase in the volume and weight of the ISB is noted. Subsequently this enlargement becomes more striking until the seventh day when a 2-fold increment is noted concurrently with a pronounced thymic atrophy. There is a regression of the ISB weight to normal values by the tenth day (Table I). Cortisone has a similar effect upon hamster brown fat located elsewhere (*i.e.,* ventrally to the scapulae, in the posterior cervical region and within the axillae).

The peak of ISB hypertrophy which is consequent to cortisone therapy occurs 7 days following administration of the hormone. There is some retardation of somatic growth under the influence of the hormone, cortisone-treated hamsters weighing after 7 days about 21 g as compared to 29 g control weight. Nevertheless, an absolute increase in brown fat in the treated groups is evident when calculated in terms of mg/100 g of body



GRAPH 2. Percentage of white fat cells within ISB according to body weight.

weight (Table I) or in terms of the amount of the fat per animal irrespective of body weight. While hamsters of all ages exhibit some measure of ISB enlargement, the degree of reactive hypertrophy diminishes with increasing body weight. Animals above 80 g respond with 25% ISB hypertrophy as compared to 200-300% in the 20 g hamster.

Histologically, there is no evidence of cellular hyperplasia to account for the rapid elevation in weight. The individual brown fat cells, however, show a striking increase in diameter achieving an average of 28 μ 7 days after cortisone injection, in contrast to normal values of 18 μ. The lipid vacuoles coalesce and the nucleus is compressed peripherally (Fig. 1). There is no microscopic evidence of undue congestion or edema to account for the elevated weight. In fact, there is a significant decrease in water content of the ISB during the period of cortisone-induced hypertrophy.

A reduction of doubtful significance in the weight of the inguinal white fat pad is produced by cortisone. Similarly, microscopic examination discloses no variation in white fat cell diameter as a consequence of cortisone administration.

Cortisone-treated mice submitted to a similar procedure also show an appreciable ISB hypertrophy as compared to control groups. The increase in weight, however, is not as striking as in the hamster, and the peak of response occurs on the fourth day (Table I).

Discussion. The morphologic and distributive distinctions between brown and white fat have stimulated considerable speculation as to possible functional divergency between the two forms of lipid tissue(5,6). Significant biochemical differences have also been recorded(7). The affinity of the MEF_1 virus for brown fat can be accepted as further evi-

TABLE I.
Effect of Cortisone upon Weight of Interscapular Brown Fat and Inguinal White Fat.

	Days after cortisone injection			
	2	4	7	10
Interscapular brown fat				
Hamsters (cortisone)	373* ± 13.6†	433 ± 25.6	632 ± 36.5	369 ± 46.1
" (control)	244 ± 14.6	259 ± 13.8	304 ± 17.4	320 ± 12.9
Mice (cortisone)	826 ± 59.4	928 ± 31.1	789 ± 47.7	686 ± 35.8
" (control)	665 ± 36.6	671 ± 18.6	637 ± 37.5	621 ± 26.7
Inguinal white fat				
Hamsters (cortisone)	250 ± 11.2	229 ± 15.4	328 ± 23.4	399 ± 20.9
" (control)	196 ± 23.7	223 ± 17.8	367 ± 29.2	510 ± 38.9

* mg/100 g body wt. † Stand. errors.

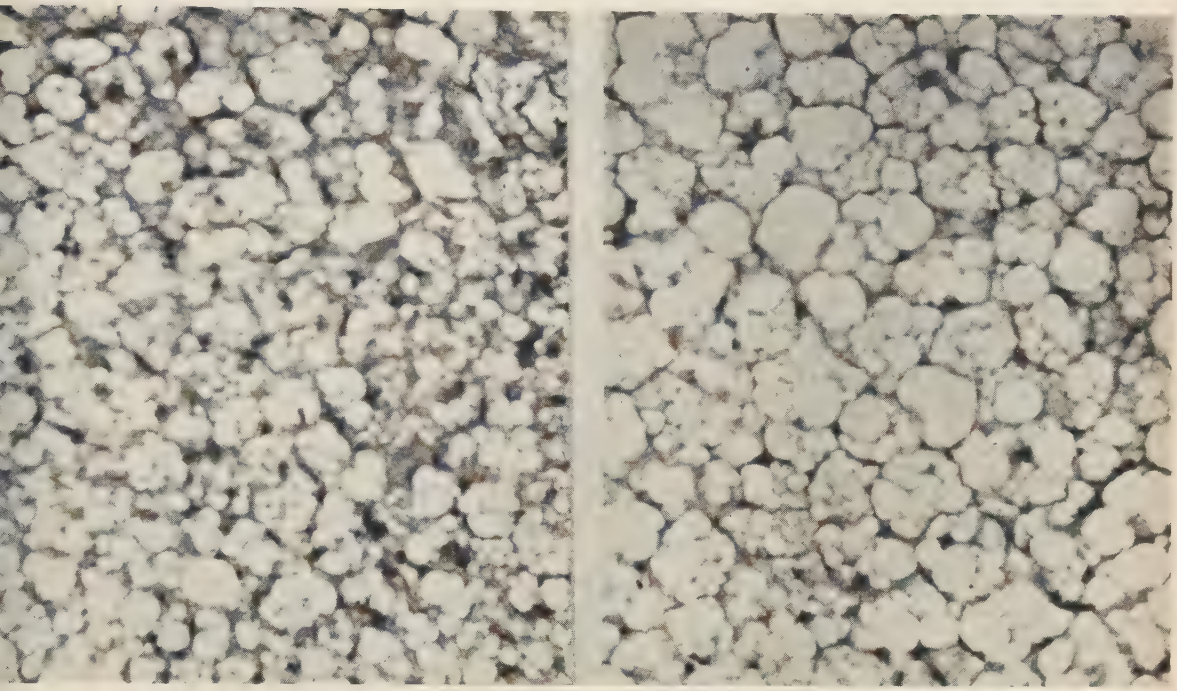


FIG. 1. (Left) Microphotograph (× 70) showing interscapular brown fat in the normal hamster. (Right) Microphotograph (× 70) showing increased cell diameter, coalescence of lipid vacuoles and peripheral migration of nuclei of interscapular brown fat in the cortisone-treated hamster.

dence of a profound distinction between the two tissues.

The effects of cortisone, described in this paper, indicate a possible relationship between brown fat and adrenocortical function. Selye and others(8,9) have noted that ACTH or stress result in a rapid reduction in ISB size. Other observers have described a primary increase in brown fat following ACTH administration(10). Antopol(11) has observed an atrophy of the ISB in mice following large doses of cortisone. The discrepancies between these reports and the observations recorded herein may be attributable in part to the varied time intervals of observation and possibly to differences in the ages of the animals studied.

Within the time limits of observation in the present report, there was noted a reduction of doubtful significance in white fat weight. Other authors noticed a decrease in the amount of this tissue after stress(12).

The degree of ISB hypertrophy after cortisone diminishes with increasing age (body weight). The reduced responsiveness is partially explained by the progressive infiltration of white fat cells within the ISB, thus decreasing the reactivity of the structure. Investigations in progress in this laboratory indicate that other factors *e.g.*, testicular activity, may play a role in mitigating cortisone-responsiveness of brown fat in older animals. Further work on the physiology of hamster brown fat, now in progress, suggests a seasonal variation in the weight and a reactivity of this tissue to a wide range of endocrine disturbances.

Summary. 1. Beyond an early age (about 3 weeks), the proportion of brown fat within the hamster maintains a relative constancy. In contrast, the ratio of white fat to body weight increases precipitously with age until full maturity of the hamster. 2. Cortisone treatment results in a prompt hypertrophy of brown fat in hamsters and mice, reflected microscopically by measurable expansion of cell diameter. White fat is possibly somewhat reduced in weight. 3. The reactive hyper-

trophy of brown fat in cortisone-treated hamsters diminishes with increasing age, apparently caused in part by the increasing numbers of unresponsive white fat cells within the borders of brown fat masses.

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Effect of Ergotamine Tartrate Upon Systemic and Peripheral Blood Sugar in Rats.* (20835)

RACHEL COSGROVE. (Introduced by Raymond W. Cunningham.)

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Shepherd and Buchanan(1) have reported the lowering of blood sugar values by ergotamine tartrate. Their blood sugar determinations were made on peripheral blood samples obtained from the tip of the rat tail. Evidence has been obtained in this laboratory to the effect that the tail blood (peripheral blood) sugar level after ergotamine tartrate treatment is not representative of the systemic blood sugar level.

In the course of blood sugar determinations on rats treated with ergotamine tartrate, it was noted that the powerful vasoconstrictive action of this agent made it difficult to bleed the rats from their tails. To overcome this difficulty blood samples were obtained by cardiac puncture. It was found that the blood sugar determinations from heart blood samples differed greatly from determinations on blood taken from the tail. Comparisons were then made between tail blood and heart blood samples taken at the same time. The results indicated a great divergence in the blood sugar level of systemic and peripheral blood. Komrad and Loew(2) have suggested that certain other vasoconstrictive agents such as Pitressin, Pitocin, and ephedrine may possibly cause a lowering of epinephrine-induced hyperglycemia by prevention of an even distribution of this hyperglycemia due to circulatory changes.

Methods. All animals used in these experiments were normal adult male Wistar rats ranging in weight from 182 to 355 g. No animal was used more than once. After a fasting period of 16 hours, the animals were placed in bleeding cages, warmed by means of a heating lamp to facilitate bleeding, and control samples taken using potassium oxalate as an anticoagulant. Control blood samples were taken only from the tail, because the high incidence of mortality following cardiac puncture outweighed the information obtained by heart blood controls. Moreover, the lack of significant difference between heart and tail blood samples subsequently demonstrated in the starch control group justified this procedure. The animals were divided into groups of 8 or 9 rats for treatment. One and one-

* Published with the approval of Dr. James H. Williams, Director of Chemical and Biological Research.

The author is indebted to Dr. William D. Gray for the statistical analysis of the data and to Miss Mary C. Clark for her assistance in preparation of this manuscript.

TABLE I. Effect of Ergotamine Tartrate, Insulin, and 2% Starch Solution on Blood Sugar Values in the Rat. 8 rats in each group.

Compound	Avg blood sugar in mg %: Control,† Tail blood	1½ hr after treatment: Tail blood	1½ hr after treatment: Heart blood
Ergotamine tartrate	92.4±4.3*	37.4±3.5*	101.0±3.6*
Insulin (control)	81.9±4.7	37.5±3.6	46.3±3.7
2% starch sol. (control)	92.4±3.3	82.4±2.5	93.5±2.8

* Stand. error of mean.
† Blood sample taken after 16 hr fast, before administration of compound.

half hours after the treatment, blood samples were taken by both methods. Blood sugars were determined by the Shaffer-Hartman-Somogyi method(3). The effect of ergotamine tartrate *per se* on blood sugar levels was investigated as follows. Three groups comprised this experiment. One group was given intraperitoneally 2.5 mg/kg of ergotamine tartrate suspended in 2% starch solution. Two groups served as controls: one group received 0.75 units/kg of insulin intraperitoneally as hypoglycemic controls, while the other was treated with 0.5 ml/100 g intraperitoneally of the starch suspending vehicle. Two groups of rats were used to investigate the effect of ergotamine tartrate on the hyperglycemic response to epinephrine. One group received 2.5 mg/kg intraperitoneally of ergotamine tartrate suspended in 2% starch solution, followed after a 20-minute absorption period by 0.2 mg/kg of epinephrine hydrochloride subcutaneously. The control group received 1.0 ml/100 g of 2% starch solution intraperitoneally, followed by 0.2 mg/kg of epinephrine hydrochloride subcutaneously 20 minutes later.

Results. The effects of ergotamine tartrate, insulin, and starch upon tail and heart blood sugar values are summarized in Table I. The control blood sugar values of the 3 groups do not differ at the $P = 0.01$ level of significance(4). With ergotamine tartrate treatment no difference of statistical significance could be demonstrated between control tail blood and 1½ hour heart blood sugar determinations. The 1½ hour tail blood was significantly less than both at the $P = 0.01$ level of significance.

The results with insulin on the other hand may be taken as a true blood sugar lowering effect. In this group the 1½ hour tail and heart blood sugar values are not different at the $P = 0.01$ level of significance, but both are significantly less than the control blood sugar values. At 1½ hours, the tail blood sugar determinations of rats receiving 2.5 mg/kg of ergotamine tartrate and rats injected with 0.75 unit/kg of insulin are the same; but the insulin heart blood sugar differs significantly at $P = 0.01$ from both its ergotamine and starch controls.

Neither tail nor heart blood sugar values differ significantly from the control determination in the group receiving only the 2% starch solution.

The effect of 0.2 mg/kg of epinephrine hydrochloride on the blood sugar picture of rats treated with 2.5 mg/kg of ergotamine tartrate in 2% starch solution is summarized in Table II. It is quite evident that inferences regarding the effect of ergotamine tartrate on epinephrine-induced hyperglycemia depend greatly on the source of the blood sample. The hyperglycemic response of the heart blood was significantly greater than the pretreatment control at the $P = 0.01$ level of significance, but was only barely significantly less than the corresponding starch controls at $P = 0.05$. With respect to systemic blood, inhibition of epinephrine hyperglycemia by ergotamine tartrate was not very striking.

In contrast to heart blood, tail blood sugar levels appeared to indicate a complete blockade by ergotamine tartrate of the hyperglycemic response to epinephrine. The 1½ hour blood sugar level was less than the pretreatment control, and was very significantly less than the corresponding starch controls at the $P = <0.001$ level of significance. In the starch-treated group, the epinephrine-induced hyperglycemia was identical in both tail and heart blood.

Summary. Under the conditions of this experiment, 2.5 mg/kg of ergotamine tartrate, intraperitoneally, had a differential effect on the peripheral and systemic blood sugar level of rats. Tail blood sugar levels were low, while blood obtained from the same animals

TABLE II. The Effect of Ergotamine Tartrate and 2% Starch Solution on Epinephrine-Induced Hyperglycemia in the Rat.

Compound	No. of rats/group	Avg blood sugar in mg %: Control,† tail blood	1½ hr after epinephrine‡: Tail blood	1½ hr after epinephrine‡: Heart blood
Ergotamine tartrate	9	86.1 ± 2.6*	53.7 ± 4.8*	154.0 ± 5.0*
2% starch sol. (control)	8	85.3 ± 3.7	212.1 ± 10.6	230.8 ± 10.1

* Stand. error of mean.
† Blood sample taken after 16 hr fast, before admin. of compound.
‡ Dose 0.2 mg/kg of epinephrine hydrochloride subcut. 20 min. after admin. of ergotamine tartrate or 2% starch sol.

by cardiac puncture exhibited normal glycemic levels. This discrepancy is probably related to the prolonged vasoconstrictive action of ergotamine tartrate and the resultant interference with normal peripheral circulation. It would appear that 0.2 mg/kg of epinephrine hydrochloride had little effect on the rat peripheral circulation since the hyperglycemic response in the starch-treated controls was equally distributed between systemic and peripheral blood. The results with insulin indicated that a true hypoglycemic agent lowered both tail and heart blood sugar values to the same extent. Slight inhibition of epinephrine-induced hyperglycemia by ergotamine tartrate was evident in heart blood, but appeared complete in tail blood, a reflection no doubt of the impairment of peripheral circulation by ergotamine tartrate. These results indicate very strongly that systemic, not peripheral blood, is the better indicator of drug effects on the blood sugar level.

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In vitro Study of Antifungal Activity of Nitrostyrenes.* (20836)

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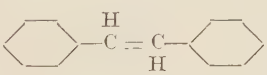
The discovery by Elson(1) of the fungistatic activity of propamidine (p,p'trimethylenedibenzamidine) against a number of pathogenic fungi led to further confirmatory investigations of other aromatic diamidines(2-6) such as pentamidine (p,p'-pentamethylenedibenzamidine) and stilbamidine (p,p'-stilbenedicarboxamidine) and to clinical trials in the treatment of systemic mycotic infections (7-12). Singular success was obtained with the use of stilbamidine in the therapy of North American blastomycosis. Curtis and Harrell(10) described 2 cases of North American blastomycosis that responded to treatment by large doses of diethylstilbestrol and noted the similarity of the chemical structures of stilbamidine and diethylstilbestrol. It was considered worthwhile to screen for antifungal activity other compounds with

* This investigation was supported by the Research and Development Division, Office of the Surgeon General, Department of the Army. Compounds tested were obtained from the Upjohn Co., the William S. Merrell Co. and the Dow Chemical Co.

TABLE I. Antifungal Activity of Nitrostyrenes. Lowest effective concentration in mg of compound per ml medium.

	β-Nitrostyrene	p-methoxy-β-nitrostyrene	p-acetoxy-β-nitrostyrene	β-methyl-β-nitrostyrene	p-methoxy-β-methyl-β-nitrostyrene
Trichophyton mentagrophytes	.1	.1	.1	.001	.001
rubrum	.01	.01	.01	.001	.001
schoenleini	.1	.1	.1	.001	.001
violaceum	.1	.1	.1	.001	.001
ferrugineum	.1	.1	.1	.001	.01
tonsurans	.1	.1	.1	.01	.001
Microsporum gypseum	.1	.1	.1	.001	.01
audouini	.001	.01	.01	.001	.001
canis	.1	.1	.1	.001	.01
Epidermophyton floccosum	.1	.1	.1	.001	.001
Cladosporium wernecki	.1	.1	.1	.01	.01
Hormodendrum pedrosoi	.1	.1	.1	.01	.01
Monosporium apiospermum	.1	.1	.1	.01	.001
Nocardia asteroides	>.1	>.1	.1	.1	.1
Candida albicans	.1	.1	1.0	>.1	.1
Cryptococcus neoformans	.01	.01	.1	.001	.01
Sporotrichum schenckii	.01	.01	.01	.01	.001
Blastomyces dermatitidis	.001	.01	.01	.001	.001
braziliensis	.01	.01	.001	.001	.001
Histoplasma capsulatum	.1	.1	.1	.001	.001
Alternaria sp.	>.1	>.1	>.1	.1	.1
Penicillium sp.	>.1	>>.1	>>.1	.1	.1
Aspergillus niger	>.1	>.1	>.1	.1	.1

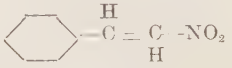
chemical formulae related to the stilbene nucleus:



Methods and materials. Antifungal activity was measured by the serial dilution agar slant method as described previously in another paper(5). This consisted essentially of setting up serial dilutions of the compound being tested in Sabouraud's dextrose agar slants in concentrations of 1 mg per ml, 0.1 mg per ml, 0.01 mg per ml, and 0.001 mg per ml medium. If complete inhibition occurred in all the tubes, the dilution was extended to 0.0001 mg per ml and 0.00001 mg per ml medium during repeat experiments. The pH of the media was determined. The solvent used was acetone so that additional acetone controls were made by adding 1% acetone to the Sabouraud's dextrose agar. The experiments were all done in duplicate and repeated when considered necessary. The test organisms used were strains of pathogenic fungi maintained in the stock collection of the Department of Dermatology of the University of Michigan Hospital. Equal amounts of suspensions of the organisms in physiological saline solution were inoculated on the agar slants. Growth was observed at room temperature and the end-point was determined at the lowest concentration of the compound where complete inhibition of the growth of the organism occurred. The 53 compounds available for the screening survey were aromatic ethylenes and included a number of cinnamic acid derivatives.

Results. In order to eliminate compounds that do not exhibit any significant antifungal powers, preliminary screening procedures were performed using as test organisms 5 fairly representative organisms: *Trichophyton rubrum, Microsporum audouini, Candida albicans, Cryptococcus neoformans,* and *Blastomyces dermatitidis.* Compounds which showed promising activity against these 5 species were tested against the remaining pathogenic fungi.

It became apparent from these preliminary results that a group of compounds belonging to the nitrostyrenes possessed a high degree of activity against pathogenic fungi. The activity of the group was higher than the other compounds tested, including stilbamidine and propamidine, whose clinical efficacy has already been demonstrated. The similarity in structure between the nitrostyrenes and the stilbene nucleus can be seen from the structural formula for β-nitrostyrene.



These 5 nitrostyrenes were 1) β-nitrostyrene, 2) p-methoxy-β-nitrostyrene, 3) p-acetoxy-β-nitrostyrene, 4) β-methyl-β-nitrostyrene and 5) p-methoxy-β-methyl-β-nitrostyrene. Their *in vitro* activity against the growth of pathogenic fungi is shown in Table I.

The literature contains some references to the antifungal properties of the nitrostyrenes. Horsfall(13) pointed out, "the excellent fungicidal and reasonably good protective powers on foliage" of β-nitrostyrene and β-methyl-β-nitrostyrene. McGowan, Brian, and Hemming(14) studying the fungistatic activity of ethylenic and acetylenic compounds, found a number of nitrostyrenes effective against *Botrytis allii, Fusarium graminearum,* and *Penicillium digitatum.* One of the components of essential oils reported to be fungistatic by Vilanova and Casanovas(15) was isoeugenol, p-hydroxy-o-methoxy-β-methylstyrene.

Summary. In screening 53 compounds with chemical formulae related to the stilbene nucleus for antifungal activity by the serial dilution agar method, a number of nitrostyrenes were found to exhibit a high degree of activity against pathogenic fungi.

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Some Factors Affecting Resistance of Mice to Experimental Histoplasmosis.* (20837)

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Relatively little information is available concerning the factors which are involved in innate and acquired immunity in histoplasmosis. Campbell and Saslaw have reported that white male mice were more susceptible to fatal infection than female mice(1). Salvin described a marked increase in resistance of mice to intracerebral challenge 6 days after immunization with acetone-dried yeast phase *H. capsulatum*(2). Farrell, Cole, Prior, and Saslaw have shown that although dogs inoculated intratracheally with mycelial phase organisms developed no clinical disease, they were resistant to subsequent challenge with mycelial phase plus cortisone which combination produced severe disease in control dogs (3).

It is the purpose of this report to describe the effects of age, sub-lethal infection and immunization on the relative resistance to fatal infection.

Materials and methods. White Swiss male mice were used throughout these studies. Intraperitoneal inoculations of 0.7, 7, 35, 70, 110 or 140 million yeast phase organisms† in 0.5 ml of 5% hog gastric mucin were employed as previously described(1). The mice were observed for a 30-day period, and necropsy performed on those dying during this interval. For immunizing antigens, thrice-washed yeast phase cultures were suspended in saline in concentrations of 35, 7, and 0.7 million organisms per 0.5 ml. These suspensions were then killed, as determined by subculture, by immersion in a 56°C water bath for 2 hours.

Results. Exp. 1 (Relation of age to susceptibility). A total of 235 mice were used in this preliminary experiment. The variation in numbers in each group was determined by the availability of mice in the designated age groups. As can be seen in Table I, a marked decrease in fatalities was observed in the mice of the 14- and 24-week-old groups (40-44%) as compared to the 80 to 88% mortalities in the 6-, 8-, and 12-week-old mice. The sharpest difference occurred between the 12- and 14-week mice. This was apparently an age rather than weight differential since there was only about a gram difference in average weights between these groups (Table I).

TABLE I. Comparative Susceptibility of Male Mice to Fatal Histoplasmosis According to Age.

Age (wk)	Avg wt (g)	Fatalities	
		No.	%
6	17	20/25*	80
8	19	67/84	79.7
12	23	22/25	88
14	24	23/52	44.2
24	27	10/25	40
6†	17	0/25	0

* Numerator = No. of deaths. Denominator = Total No. inoculated with 35 million yeast phase *H. capsulatum* in 0.5 ml mucin.

† Mucin control—Inoculated with 0.5 ml mucin only.

Exp. 2 (Comparison of 8- and 14-week-old mice). To investigate further the results obtained in Exp. 1, 2 groups of mice, 8 and 14 weeks of age, were simultaneously challenged with varying doses of organisms as can be seen in Table II. Again significantly greater re-

TABLE II. Comparative Fatal Histoplasma Infections in 8- and 14-Week-Old Mice.

Dose (million)	8-wk mice deaths		14-wk mice deaths	
	No.	%	No.	%
140	43/43	100	16/20	80
70	46/50	92	11/20	55
35	67/84	79.8	9/20	45
7	14/42	33.3	4/20	20
.7	4/53	7.5	1/20	5
Mucin controls	0/25	0	0/20	0

* This investigation was supported by a research grant from the National Institute of Health, Public Health Service.

† Strain G-13, a recent isolate from spontaneous histoplasmosis in a dog kindly supplied by Charlotte Campbell of Army Medical Center, Washington, D.C.

TABLE III. Effect of Heat-Killed Yeast Phase Antigen on Susceptibility of Histoplasma Infection.

Antigen dose (million)	Challenge dose (million)* 35 deaths No.	%	110 deaths No.	%
35	9/43	20.9	15/46	32.5
7	28/94	29.7	25/53	52.8
.7	16/51	31.3	26/46	56.5
Saline control†	21/50	42.0	37/50	74.0

* Admin. 6 wk after "immunization."
† .5 ml saline given instead of antigen.

sistance was noted in the older mice receiving the larger inocula of 140, 70, and 35 million organisms.

Exp. 3 (Effect of immunization). Three groups of 8-week-old mice were vaccinated with 0.7, 7, or 35 million heat-killed organisms and challenged 6 weeks later with 35 or 110 million cells. The results, as can be seen in Table III, indicate that significantly increased protection was noted in the mice receiving the 35 million dose antigen. A lesser degree of protection was afforded by the 7 and 0.7 million antigen dose.

TABLE IV. Results of Reinfection of Mice Following Initial Sub-Lethal Infection.

Initial infection Dose (million)	Deaths No.	%	Reinfection Dose (million)	Deaths No.	%
35	94/109	86	35	2/15	13
7	25/ 74	34	35	4/49	8
.7	6/ 73	8	35	14/67	21
M*	0/ 48	0	35	21/48	43.7
.7	4/ 50	8	110	18/46	39.2
M	0/ 50	0	110	37/50	74.0
7	14/ 42	33.3	70 (killed)	0/28	0

* M = Mucin control.

Exp. 4 (Reinfection studies). In an effort to determine the effect of initial sub-lethal infection on the resistance of mice to subsequent reinfection, the following study was conducted. Mice which survived challenges of 0.7, 7, and 35 million organisms at 8 weeks of age were inoculated with 35 or 110 million organisms 6 weeks later. As can be seen in Table IV, a significant decrease in mortality was observed in the mice previously surviving infection. It was noted during the course of this experiment that the deaths in the reinfection group usually occurred within the first 4 days. In contrast, after primary infections, most fatalities were between the 7th to 22nd days. In order to determine whether this was a sensitization or shock phenomenon, 70 million dead organisms in mucin were inoculated into a group of 28 mice which had previously survived infection, but none succumbed to the challenge (Table IV). Similarly none of 42 previously infected mice were affected by 0.5 ml of a 1:100 dilution of histoplasmin, intraperitoneally. The time relationship of deaths in the various categories studied in this project are presented in Table V.

Discussion. The data presented in these studies suggest that white Swiss male mice 8 to 12 weeks of age are more susceptible to experimental histoplasma infections than those 14 weeks or older. This sharp distinction may be related in part to the fact that sexual maturity is not attained by male mice until after 8 to 12 weeks(4). The increased resistance observed after this developmental phase may be compared to observations in human beings. Parsons and Zarafonetis noted that the incidence of human histoplasma infections was highest during the first year of life and after falling off rapidly reached another peak after the 5th decade(5). Their observations also noted that up to, and including the age of 10, the incidence was the same in both sexes, but after that age the ratio was about 7 to 1 in favor of males. Our previous studies with sexually mature mice also showed a greater susceptibility in males (1). This is in contrast to Salvin's report that no differences in susceptibility were noted between male and female mice(2). However, it is of interest to note that he used sexually immature 35-day-old mice. Additional studies to elucidate further the sex and maturity factors are in progress in this laboratory.

That mice surviving previous infections are more resistant to subsequent reinfection with the same organism is also suggested by these studies. It is recognized that the survivors might represent a more naturally resistant group. However, increased protection was also noted in mice receiving the smaller doses which had previously resulted in a low fatality rate. Actually a different pattern of response

TABLE V. Time of Deaths of Mice Following Reinfection with *H. capsulatum.*

1st inoculum	2nd inoculum	No. deaths	% of deaths after days: 1–4	5–8	9–12	13–16	17–20	21–24	24–29
Live cells (.7-35 million)	Live cells (7-110 million)	37/177	17.0	.6	1.7	.6	1.2	0	0
Dead cells (.7-35 million)	*idem*	122/333	1.7	1.3	15.0	12.9	4.9	1.0	0
Normal saline	*idem*	326/541	1.6	11.3	20.3	13.8	6.2	5.2	1.6
Live cells (.7-35 million)	Dead cells (70 million)	1/ 42	0	2.4	0	0	0	0	0
idem	Histoplasmin (.5 cc of 1:100 dilution I.P.)	0/ 42	0	0	0	0	0	0	0
Mucin	Mucin	0/ 25	0	0	0	0	0	0	0

was noted in the reinfection group in that most of the fatalities occurred within 4 days after challenge as compared to the predominance of deaths between 7 to 22 days after primary challenge. Heat-killed antigens or histoplasmin did not induce such reactions in previously infected mice. The mechanism of this phenomenon is being investigated in this laboratory. The introduction of heat-killed antigen resulted in a significantly lowered mortality rate following subsequent challenge 6 weeks later. It is of interest to note that Salvin, using the intracerebral route, found that protection following immunization was greatest 6 days later, animals at that time withstanding 320 $LD_{50's}$ as compared to 10 $LD_{50's}$ at 24 days. This latter period is even less than the 6-week interval which was employed in these studies.

Summary. White Swiss male mice 8 to 12 weeks of age were more susceptible to intraperitoneal inoculations of yeast phase *H. capsulatum* than mice 14 weeks or more in age. Mice surviving previous sub-lethal infections were more resistant to subsequent challenge 6 weeks later. Vaccination with heat-killed yeast phase antigens resulted in a decrease in the number of fatal infections following massive challenge 6 weeks later.

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Ion-exchange Chromatography of Oxytocin, Arginine-vasopressin, and Lysine-vasopressin.* (20838)

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In the course of the isolation of hormones from beef and hog posterior pituitary glands, it was found that oxytocin prepared from beef sources could not be differentiated from that obtained from hog by the technics employed (1). Vasopressin, however, had a different composition in these two species. The vasopressin prepared from beef glands was differentiated from that found in hogs in that the former contained arginine; this material has been termed *arginine-vasopressin*. Hog glands have yielded a vasopressin characterized by the presence of lysine in place of the arginine found in vasopressin from beef sources, and this has been termed *lysine-vasopressin*(2,3).

Previous attempts in this laboratory to separate and define these preparations as chemical entities have involved principally the use of countercurrent distribution technics (2,4) and zone electrophoresis on various supporting media(5,6). Filter paper electrophoresis has proved to be an extremely useful analytical tool for the routine detection of oxytocin; however, the method does not offer a critical separation of lysine- and arginine-vasopressin since the isoelectric point of lysine-vasopressin is only slightly lower than that of arginine-vasopressin, and their pH-electrophoretic mobility curves are similar(7). Hence there is a need for another method of separating the 2 known vasopressins on an analytical scale. For this purpose, an investigation has been made of the chromatographic behavior of these 3 hormones on the carboxylic acid ion exchange resin Amberlite IRC-50 (XE-64). This resin has been found to be suitable for the chromatography of cytochrome c(8), pancreatic ribonuclease(9), egg lysozyme(10), and spleen lysozyme(11).

Methods. Chromatography was carried out on 0.9 x 30 cm columns of ion exchange resin Amberlite IRC-50 (XE-64). The preparation of the resin and operation of the column was carried out according to the procedures of Hirs, Moore, and Stein(9) and Tallan and Stein(10). The rate of flow of solvent through the column was 1.5 ml per hour. 0.5 ml fractions of the effluent were collected for analysis. Sodium phosphate buffer was used as the eluting agent; the buffer was saturated with chloretone as a preservative. Analysis of effluent fractions was carried out with the modified Folin reagent(12). These fractions were collected in 13 x 100 mm pyrex test tubes; 3 ml of reagent C were added followed by the addition of 0.3 ml of dilute Folin reagent. Readings were made at 750 mμ in the Beckman spectrophotometer. Oxytocic activity was determined by the method of Coon (13) and pressor activity was measured on cats anesthetized with sodium phenobarbital. *Oxytocin* in these studies was prepared from a crude material of 50 units per mg supplied by Armour and Co. The material was extracted with *sec*-butyl alcohol-acetic acid; the butyl alcohol phase was then washed with phosphate buffer(14). After countercurrent distribution in *sec*-butyl alcohol-acetic acid this material had a potency of approximately 500 oxytocic units per mg. Arginine-vasopressin was obtained from a product extracted from beef glands which had an activity of 50 pressor units per mg. This product was subjected to electrophoresis, using procedures previously described(6), on a cellulose‡ supporting medium(7). A pyridine-acetate solu-

* Appreciation is expressed to the Lederle Laboratories Division, American Cyanamid Co., for a research grant which has aided greatly in this work. Acknowledgement is also made to Armour and Co., who have made available crude extracts of pituitary glands, and to Parke, Davis, and Co. for generous gifts of posterior pituitary powder.

† Deceased, Oct. 9, 1953. This manuscript was virtually completed at the time of the tragic death of Dr. Taylor (V. du V.).

‡ Solka-Floc, SW-40-A, Brown Co., New York City.

tion at pH 5.7, $\Gamma/2 = 0.1$, was employed as a buffer. The active fractions were collected, filtered, and lyophilized to dryness; the arginine-vasopressin so obtained had a potency of 400 pressor units per mg dry weight. Lysine-vasopressin was similarly prepared from a starting material with a potency of 50 pressor units per mg that was obtained from hog sources; the final product had a potency of 210 pressor units per mg.

Results. Preliminary equilibration experiments(9) were performed in a search for a suitable phosphate buffer concentration and pH level for chromatography of arginine-vasopressin; at pH 6.95 in 0.2 M sodium phosphate buffer sufficient absorption of the vasopressin occurred to insure success of a column. When arginine-vasopressin was placed on the IRC-50 column with this buffer as the eluting agent, a single peak emerged from the column at approximately 70 effluent ml. Assays for pressor activity showed a reasonable but not perfect correspondence with the Folin phenol color, indicating the presence of some inactive impurities. Lysine-vasopressin was chromatographed in a similar manner at pH 6.95 in 0.2 M phosphate buffer; it emerged at 40 effluent ml in a peak that contained the expected pressor activity. Oxytocin, being less basic than lysine-vasopressin, would be expected to precede lysine-vasopressin on elution from the column. Accordingly, 0.6 mg of oxytocin, 0.6 mg of lysine-vasopressin, and 0.7 mg of arginine-vasopressin were dissolved in 0.5 ml of the buffer and chromatographed in the usual manner. The analysis of effluent

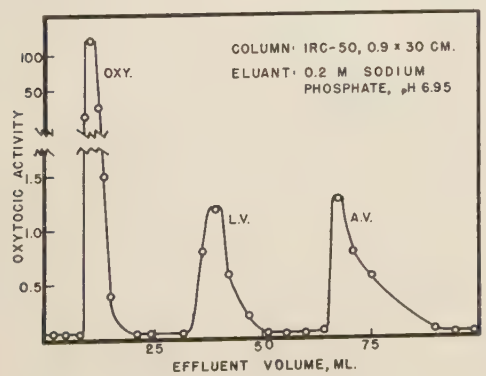

FIG. 1. Chromatography of a mixture of oxytocin (Oxy.), lysine-vasopressin (L.V.) and arginine-vasopressin (A.V.).

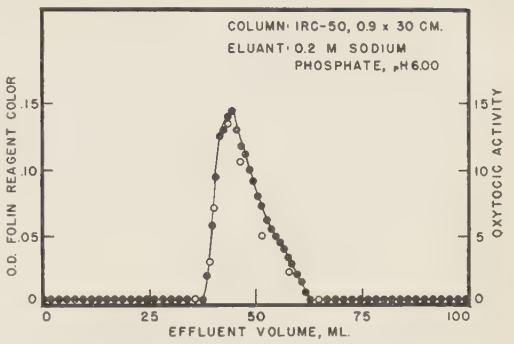

FIG. 2. Chromatography of 0.8 mg of oxytocin. ●, Folin reagent color; ○, oxytocic activity.

fractions for their oxytocic activity is shown in Fig. 1. Oxytocin emerged after the first column volume without any retardation. Lysine-vasopressin emerged at 40 ml, while arginine-vasopressin was retarded to the extent that its peak appeared at 70 ml. While this experiment demonstrated a separation of the three known posterior pituitary hormones, it does not represent a true chromatography of oxytocin since this peptide emerged as a narrow peak after the first column volume.

Chromatography of oxytocin was accomplished on IRC-50 by employing 0.2 M sodium phosphate buffer at pH 6.00. Under these experimental conditions oxytocin appeared with a peak at 45 effluent ml, as shown in Fig. 2. Oxytocic assays performed on several fractions showed a close correspondence between oxytocic activity and phenol color. *Synthetic oxytocin*§(15) was found to appear in the same position on the chromatogram and the shape of the curve was similar to that of Fig. 2. When a mixture of 0.37 mg of natural oxytocin and 0.34 mg of synthetic oxytocin was chromatographed under the same conditions the results were again essentially the same as those of Fig. 2.

Discussion. IRC-50 resin appears to possess a high degree of resolution in that it can separate two highly basic peptides of similar structure(16,17), lysine-vasopressin and arginine-

§ The *synthetic oxytocin* is a sample of the synthetic octapeptide amide with the hormonal activity of oxytocin(15). From the many comparisons of the synthetic material and natural oxytocin which have been made there appears no doubt that the synthetic product represents oxytocin.

vasopressin. Both of these hormones have been shown to possess inherent oxytocic activity(18,19). It is therefore possible to demonstrate the presence of the vasopressins by means of an assay for oxytocic activity. The fowl depressor assay of Coon(13) was used as a measure of the oxytocic activity in the present work. This method has previously been shown to give an estimate of oxytocic activity which is high by a factor of 2.7 when there is present predominant amounts of pressor activity(18). The results presented in Fig. 1 indicate that all three of the known hormone preparations derived from the posterior pituitary glands are potent oxytocic agents.

The chromatography of oxytocin required the resin column to be buffered at pH 6.00. By comparison, Hirs, Moore, and Stein reported that ribonuclease can be chromatographed at pH 6.47. Since oxytocin and ribonuclease have nearly similar isoelectric points at pH 7.7 and 7.8 respectively, the rather low pH required for the chromatography of oxytocin is probably due to the fact that oxytocin is virtually uncharged at its isoelectric point(6). Thus a sufficient degree of ionization of the molecule to enable it to react with IRC-50 is not achieved until the pH is lowered to 6.00. The oxytocin preparation examined in Fig. 2 appears to consist of a single active component in which the oxytocic activity corresponds to the phenol color. Investigation of the behavior of synthetic oxytocin on IRC-50 has shown that it occupies the same position in the chromatogram as the natural material, and that a mixture of synthetic and natural oxytocin chromatographs as a single substance.

Summary. Oxytocin, lysine-vasopressin, and arginine-vasopressin can be chromatographed on ion exchange resin IRC-50 (XE-64). Complete separation of the three hormones was achieved by passage through a column buffered at pH 6.95 with 0.2 M phosphate buffer; under these conditions lysine-vasopressin and arginine-vasopressin emerged at 40 and 70 effluent ml respectively, while oxytocin passed through without retardation. At pH 6.00, with 0.2 M phosphate buffer as the eluting agent, oxytocin, synthetic oxytocin, and a mixture of natural and synthetic oxytocin chromatographed as a single substance.

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Use of *Hyla cinerea* in Assay of Melanophorotropic Potency of ACTH. (20839)

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The nature of the relationships between ACTH and melanophorotropic hormone has not yet been satisfactorily settled(1). Either, as claimed by Sulman(2), the two represent fractions of a corticotropic complex of hormones or, as Reinhardt, *et al.*(3) believe, the melanophorotropic action of ACTH preparations is the result of contamination by melanophorotropin. Should the two hormones be shown to be identical, assays employing melanophore reactions will become increasingly important; should they be demonstrated to be separate hormones it will become increasingly important to know the melanophorotropic potency of ACTH preparations as a possible undesirable side effect in ACTH therapy(4).

Although assay methods for melanophorotropin have been published by many workers, the simplicity of Sulman's(5) technic has much to recommend it. In a search for an American frog which would fulfill the requirements of this assay, *Hyla cinerea* has been studied in this laboratory.

Materials and methods. Tree frogs (*Hyla cinerea* Schneider) were maintained in 1500 ml beakers covered with watch glasses. No more than 6 frogs were placed in a single beaker. Each beaker contained about 2 cm of tap water. Frogs were irregularly fed on *Drosophila*. Injections were made directly into the dorsal lymph sac; dosages were contained in 0.1 ml of distilled water. As gross color changes seemed to give as accurate an estimate of melanophore condition as microscopic examination, they were used exclusively. After ACTH injection the green frogs went through a regular series of color changes: green to light olive to dark olive to blackish-brown. This continuum was divided into 4 arbitrary stages, and each stage was assigned a numerical value (Table I). In statistical treatments the numerical values for all frogs at a given dose level were averaged; the resulting mean was termed the color index (CI). As a result of the rapidity of the response, time-response curves were based on readings taken at 5-minute intervals. No change in sensitivity has been noted with repeated use of the frogs, although generally 48 hours were allowed to elapse between tests. A single sample of ACTH was employed; this sample assayed at 1.3 IU/mg.

TABLE I. Numerical Values Assigned to Colors of *Hyla cinerea*.

Color	Numerical assignment
Green	1
Light-olive	2
Dark-olive	3
Brown	4

Experimental. Since no study of environmental control of color change in *Hyla cinerea* had ever been published, and such information was prerequisite to the interpretation of the assay, a preliminary analysis was carried out in this laboratory. Details of methods used will appear elsewhere(6). Light (and its absence), temperature and humidity have been shown to be the most important environmental stimuli for color change(7). In constant light *Hyla cinerea* showed no alteration of the green color; in constant darkness they tend to blanch slightly, becoming yellowish after 24-48 hours. Low temperatures (4-5°C) stimulated darkening in only ⅓ of the frogs after about 30 minutes. By keeping all frogs in beakers containing similar amounts of water, relative humidities were maintained at constant levels throughout.

Effects of ACTH on color change: ACTH was administered in doses from 0.1 to 50.0 μg/frog. Both the magnitude of the CI and the time required for the index to return to control levels were related to dose (Fig. 1). Distilled water, which was used as a control, produced a slight initial increase in CI which fell to normal in 30 minutes to 1 hour. Inspection of the curves of Fig. 1 indicates that the 30-minute response best differentiated the

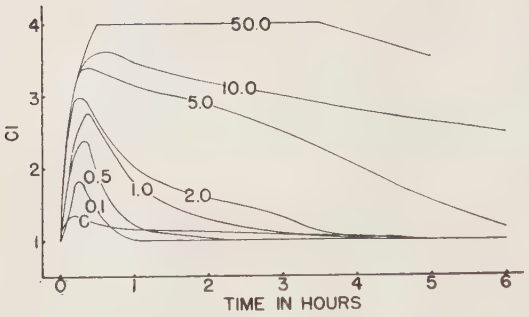

FIG. 1. Temporal responses in color change of *Hyla cinerea* following μg injections of ACTH at 0 hr. CI = color index (see text); C = distilled water controls.

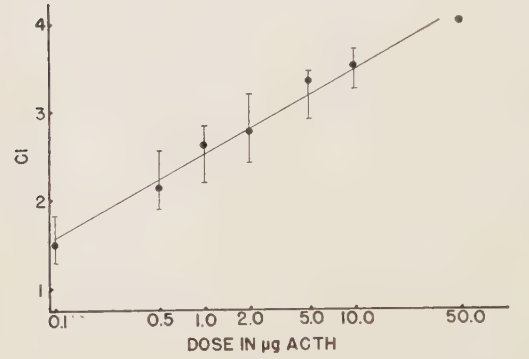

FIG. 2. Regression of color index (CI) on log dose ACTH 30 min. after injection. CI = 1.56 + 0.95 log (10 dose), by method of "least squares." Vertical lines indicate 95% confidence limits.

various levels of ACTH. The half-hour responses were directly related to log dose (Fig. 2). In a series of experiments, known doses of ACTH were administered to groups of 4 frogs each; the investigator reading the frogs was in ignorance of the sequence of the doses. In all cases it was possible to make correct determinations of administered doses.

Discussion. It is apparent from the experiments summarized on the normal control that under conventional laboratory conditions of temperature (20-24°C) and light there is little or no color change. Thus frogs may be maintained in the laboratory with no special care, obviating the difficulties of hypophysectomy necessary with Ranid frogs and the time and space necessary for light-conditioning in *Hyla arborea* or *Xenopus* tadpoles(5,8). The rapidity of the response of these frogs to ACTH preparations allows the assay of materials in less than 2 hours. The response observed with the injection of distilled water may be interpreted as the result of the release of endogenous melanophorotropic hormone or perhaps epinephrine as a result of handling, the excitement darkening of Burgers, *et al.* (9). Since this reaction is neither as great in magnitude nor in duration as that obtained with low ACTH doses employed, it does not interfere with the assay.

Summary. The lack of integumentary color changes in *Hyla cinerea* under normal laboratory conditions makes it a suitable organism for the assay of the melanophorotropic potency of ACTH. Intensity of darkening is directly related to the log of the dose.

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Nucleotide Conversions in Various Tissues of Pregnant and Tumor-bearing Mice.* (20840)

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Earlier(1,2) we reported that during the growth of implanted sarcoma 180 in mice there occurs simultaneously a decrease in blood adenosine triphosphate (ATP) and an increase in tumor tissue nucleotide. The only other of several experimental conditions tested, under which we observed a depression of blood nucleotide, was pregnancy. The present investigation is concerned, first, with the relationship between nucleotide level in the blood of pregnant mice at various gestational stages and that in embryo tissue; second, with the nucleotide levels in liver, spleen, and muscle of tumor-bearing animals, as well as in liver and spleen of pregnant mice. Since in our earlier study(2) we had found that the injection of 5-adenylic acid tended to correct the depressed ATP blood level in tumor-bearing mice, as well as the elevated nucleotide level within the tumor tissue, experiments were undertaken to ascertain the effect of injected 5-adenylic acid on the nucleotide level in spleen, liver, and tumor tissue of tumor-bearing mice, and in the spleen, liver, and embryo tissue of pregnant mice.

Materials and methods. White Swiss female mice of standard age were used throughout. In the case of the tumor experiments, the animals were implanted bilaterally with sarcoma 180, using methods now standard for this procedure. Tumors were removed 7 days after implantation. In the case of the pregnancy experiments, embryos—more correctly, fetuses—were removed at various stages of gestation; the tissue classified as "embryo" comprised the amnion, allantois, placenta, and fetus. The amniotic fluid was in each case discarded. The 16-day stage of pregnancy was arbitrarily selected for certain of the experiments on embryo tissue. The animals were killed by cervical fracture; then as quickly as possible, the tissues of interest were excised and dropped into liquid nitrogen. In the case of tumors, the central necrotic core of each growth was removed and discarded. Tissues were often taken from multiple donors of the same experimental class so as to yield a wet weight aliquot of about one g. For muscle assay, a rectangular section from the ventral abdominal musculature was used. The frozen tissues were pulverized and extracted once with a 5-ml aliquot of cold 10% trichloroacetic acid and twice with 2 ml aliquots of 5% trichloroacetic acid. Following centri-

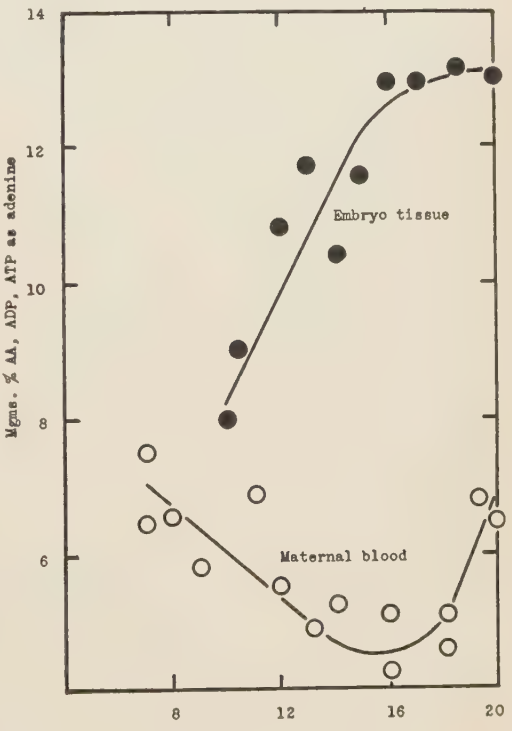

FIG. 1. Known nucleotide (AA, ADP, and ATP) in blood of mice at various stages of pregnancy, and in the embryo tissue.

* This investigation was supported in part by a research grant from the National Cancer Institute of the National Institutes of Health, Public Health Service.

TABLE I. AA, ADP, ATP, and Total Purine Levels in Liver Tissue from Normal, Tumor-Bearing, and 16-Day Pregnant Mice, with and without Adenylic Acid Pre-Treatment. Mean values, expressed as mg % adenine, given along with their standard errors. I = animals inj. intrav., one hr before sacrifice, with 5 mg 5-adenylic acid in .25 ml water. U = No inj.

	Normal		Tumor-bearing		Pregnant	
	U	I	U	I	U	I
AA	12.6 ± 1.16	12.7 ± .89	12.2 ± .87	12.3 ± .80	11.2 ± 1.07	10.7 ± .49
ADP	5.6 ± 1.56	3.9 ± 1.03	4.0 ± .83	3.3 ± .80	2.5 ± 1.00	1.6 ± .69
ATP	14.1 ± 1.18	18.6 ± 1.19*	12.2 ± .83	14.7 ± .96*	7.7 ± .93†	10.3 ± .75*
Total purine	56.8 ± 1.21	61.9 ± 1.71*	54.5 ± .95	58.1 ±1.53*	48.0 ± 1.74†	54.3 ±1.75*

* Difference between means of uninj. (U) and adenylic acid inj. (I) significant (*i.e.*, diff. between means/S. $E._{diff}$ is 2 or more.

† Difference between means of experimental group and normal group significant.

$$S.E._{mean} = \frac{\sigma}{\sqrt{n}}; \quad S.E._{diff} = \sqrt{(S.E.)^2_{control} + (S.E.)^2_{experimental}}$$

fugation, the extracts were combined, neutralized, made to 10-ml volume, treated with .5 ml of 25% barium acetate and 4 volumes of ethyl alcohol, and placed in the cold room overnight. The barium precipitates were collected by centrifugation, dissolved in dilute HCl, freed of barium with Na_2SO_4, neutralized, made to volume and assayed enzymatically for adenine due to adenylic acid (AA), adenosine diphosphate (ADP), and adenosine triphosphate (ATP), following methods described earlier (1). Total purine was determined spectrophotometrically from the 2600 Å reading.

Results. The combined adenine present as AA, ADP, and ATP in the mouse embryo is shown in Fig. 1, plotted as a function of pregnancy period. Also plotted are the data from our earlier publication(2) on the blood level of these nucleotides in pregnant mice. It is clear that decrease in blood nucleotide is related to an increase in embryo nucleotide. The nucleotide level in pregnancy blood begins a return to normal at about the 16th day of pregnancy, at which time the embryo level appears already to have reached a constant value. The rapid increase of embryo nucleotide up to about the 16th day of pregnancy may reflect the tissue differentiation pattern of fetal growth.

Table I shows the nucleotide levels in liver from normal, tumor-bearing, and pregnant mice. Although the ATP level in liver tissue from tumor-bearing animals tends to be lower than in normal animals, the difference between the means is not statistically significant, probably because of the relatively small number of determinations made and the extent of their variability. The ATP level in liver tissue from pregnant animals, on the other hand, undergoes an appreciable and very significant lowering. The total purine is depressed somewhat in the liver of tumor-bearing mice and, again, more markedly, in the liver of pregnant animals. In all cases, injection of adenylic acid raises both the ATP and the total purine levels in mice, regardless of experimental state, an effect recently demonstrated for rats(3).

Table II shows similar data for the spleen of normal, tumor-bearing, and pregnant mice.

TABLE II. AA, ADP, ATP, and Total Purine Levels in Spleen Tissue from Normal, Tumor-Bearing, and 16-Day Pregnant Mice, with and without Adenylic Acid Pre-Treatment. Mean values, expressed as mg % adenine, given along with their standard errors. Injection data identical to that of Table I.

	Normal		Tumor-bearing		Pregnant	
	U	I	U	I	U	I
AA	6.7 ± .93	5.5 ± .84	8.2 ± 1.04	6.7 ± .69	8.6 ± 1.09	5.2 ± 1.09
ADP	3.1 ± 1.24	3.4 ± 1.24	4.4 ± .93	4.5 ± 1.24	7.0 ± 2.96	2.9 ± 2.53
ATP	19.2 ± 1.21	22.8 ± .99*	14.2 ± 1.51†	17.4 ± 1.68	9.1 ± 2.38†	14.4 ± 1.98
Total purine	48.6 ± 1.20	52.0 ± 1.62	46.2 ± 1.3	48.0 ± 1.28	51.8 ± 1.55	52.0 ± 3.94

* and † See footnotes, Table I.

TABLE III. AA, ADP, ATP, and Total Purine Levels in Abdominal Muscle Tissue from Normal and Tumor-Bearing Mice, with and without Adenylic Acid Pre-Treatment. Mean values, expressed as mg % adenine, given along with their standard errors. Injection data identical to that of Table I.

	Normal		Tumor-bearing	
	U	I	U	I
AA	3.1 ± .25	3.3 ± .33	2.8 ± .30	2.9 ± .24
ADP	1.2 ± .26	.9 ± .53	1.8 ± .48	2.0 ± .64
ATP	54.7 ± 2.22	57.0 ± 2.09	49.5 ± 2.00	52.7 ± 1.93
Total purine	71.4 ± 1.19	72.0 ± 1.69	65.6 ± 1.77†	67.3 ± 1.31

† See footnote, Table I.

One observes a marked decrease in spleen ATP for both tumor-bearing and pregnant animals, but without an accompanying decrease in total purine, indicating shifts from the unidentifiable purine fraction. As in the case of liver, changes in the spleen of pregnant animals are more marked than those occurring in tumor-bearing animals. Injected adenylic acid tends to a limited extent to increase ATP and total purine in the spleen of mice from all the experimental groups.

Table III shows nucleotide levels in abdominal muscle from normal and tumor-bearing mice. Muscle tissue from pregnant mice was not studied in this connection. There is a slight decrease of total purine in muscle tissue from tumor-bearing animals, both with or without injected adenylic acid, but no significant changes are seen in the AA, ADP, or ATP levels in muscle from either normal or tumor-bearing mice. This is at variance with data reported in this connection for rats(3), where the injection of adenylic acid in large doses significantly elevated the ATP level in gastrocnemius muscle. This variance may be related to the adenylic acid dose used for rats having been 25 times greater than the dose we used for mice.

Table IV shows the effect of injected adenylic acid on the nucleotide levels in embryo tissue as well as in tumor tissue. It is clear that whereas injected adenylic acid significantly lowers the ATP level of tumor tissue, as previously reported(2), adenylic acid induces no significant change in the nucleotide level of embryos. This may mean that injected adenylic acid does not pass across the placental barrier.

Summary. In mice, a progressive lowering of blood nucleotide during pregnancy occurs simultaneously with an increase in nucleotide level for the embryos of such animals. Injection of adenylic acid into tumor-bearing mice produces a lowering of the nucleotide level in the tumor tissue, in contrast with no significant effect on the nucleotide level in embryo tissue. The liver and spleen undergo a lowering of tissue ATP as a function of pregnancy, and to a lesser extent as a function of tumor-growth. No significant alteration in muscle nucleotide is seen for mice bearing implanted tumors. The injection of adenylic acid elevates the ATP level in liver and spleen of normal, tumor-bearing, and pregnant animals, but, at least in the case of normal and tumor-bearing mice, has no effect on muscle nucleotide. Following adenylic acid injection, liver tissue from normal, tumor-bearing, and

TABLE IV. AA, ADP, ATP, and Total Purine Levels in Embryo Tissue from 16-Day Pregnant Mice, and in 7-Day Tumors (Mouse Sarcoma 180). Mean values, expressed as mg % adenine, given along with their standard errors. Injection data identical to that of Table I.

	Embryo		Tumor	
	U	I	U	I
AA	2.0 ± .18	1.8 ± .24	3.3 ± .52	3.2 ± .45
ADP	1.3 ± .30	1.0 ± .21	.8 ± .34	3.2 ± 1.04
ATP	9.0 ± .56	9.0 ± .56	11.2 ± .73	8.4 ± .88*
Total purine	28.5 ± .51	25.8 ± .77	35.4 ± 1.11	32.6 ± 1.42

* See footnote, Table I.

pregnant mice shows an increase in total purine, whereas the spleen does not.

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Pharmacology of Smooth Muscle Valve in Renal Portal Circulation of Birds.* (20841)

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The avian renal portal system, first described by Jacobson in 1822(1) was not proved functional until 1948 by Sperber(2). In the bird the external iliac vein (EIV) communicates with the internal iliac vein which drains into the renal parenchyma. The EIV also connects directly to the renal vein (RV) by a large venous shunt. Interposed at the point of communication from EIV to RV is a valve-like structure described by Spanner (3) which is of variable shape in various species of birds but which is composed of smooth muscle, and usually has a conical shape with single or multiple apical openings. When contracted, the valve would tend to divert iliac vein blood through the kidney and when relaxed, blood would pass directly into the renal vein. Sperber described the position and appearance of the valve (Fig. 1) and presumed that it regulated blood flow to the renal tubules. Sperber clearly illustrated the functional significance of the renal portal system in the chicken by demonstrating that phenol red injected into one leg appeared in excess in the urine of the ipsilateral kidney.

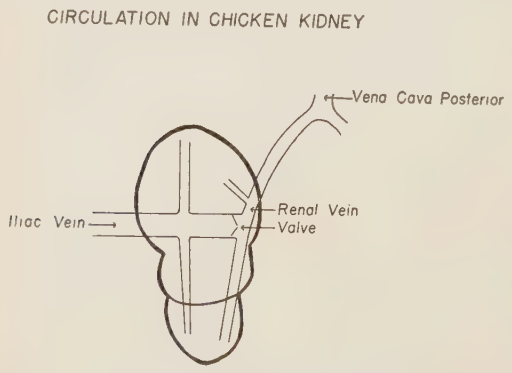

FIG. 1. Renal-portal circulation in chicken kidney.

The present report deals with the response of this valve to autonomic drugs as determined by direct recording of the response of the isolated structure suspended in a smooth muscle bath, and as estimated by the excess excretion of para-aminohippurate by one kidney when this substance was injected into the ipsilateral leg or iliac vein.

Method. In vitro. Valves were dissected from veins of freshly killed turkeys† weighing about 25 lb. The valve was suspended in oxygenated Tyrode's solution at 37.5°C in an Anderson muscle bath and attached to a light weight lever recording on a smoked drum. The bath volume was 30 ml. *PAH uptake* by chicken kidney slices was determined by the method of Cross and Taggart(4) with one modification. The final PAH concentration was 0.0013 M. Slice to medium ratios were calculated on a wet weight basis. *In vivo.* Hens were anesthetized with sodium barbital, 200 mg/kg intravenously and infused with 1 ml per minute of glucose-saline solution. Urine was collected separately thru plastic cups‡ sewed over the cloacal orifice of each ureter and the recovery of PAH in the urine

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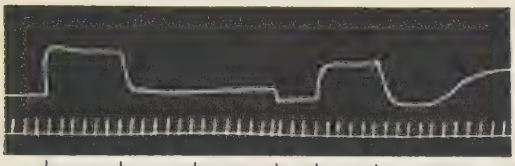
† Generously donated by Mr. Fred Plume, Regional Poultry Market, Syracuse.

‡ Designed and made by Mr. E. J. Toner.

FIG. 2. Response of excised turkey vein valve to autonomic drugs.



		mg
Ach	= Acetylcholine	.05
Atr	= Atropine	.50
Off	= 10 min. wait after wash	
H	= Histamine	.025
E	= Epinephrine	.010
Time	= 10 sec.	

was assessed when administered to the renal portal system unilaterally. The amount of PAH in the urine on the injected side when compared with the amount recovered from the control side gives an index of the magnitude of the functional renal portal circulation at the time of injection and hence reflects the tonic state of the valve. When the valve is tightly closed the recovery of unilaterally injected PAH would be much greater from the urine of the injected side than from the control kidney in the same animal. With the valve widely open one would expect to find nearly equal amounts of PAH excreted by the two kidneys.

Results. In vitro. Fig. 2 shows that the excised smooth muscle valve of the turkey renal venous system responded by contraction to acetylcholine. The increased tonus was immediately reduced by atropine, and further contraction by acetylcholine was prevented by the presence of atropine. Histamine contracted the valve and this contraction was relaxed by epinephrine and also by norepinephrine. In other experiments the response of the smooth muscle to acetylcholine was potentiated by physostigmine.

In vivo. In intact hens under urethane anesthesia, PAH injected into one leg was recovered in approximately equal amounts in the urine of the two kidneys, indicating that under these circumstances little or no iliac vein blood was diverted into the renal portal system. Under barbital anesthesia on the other hand, PAH similarly administered was recovered in great excess from the ipsilateral kidney, ranging from 2 to 15 times the excretion of the control kidney, and indicating that the portal shunt must have been closed by contraction of the valve.

Assuming, on the basis of the *in vitro* experiments, that the valve contracts under cholinergic influence, several experiments were carried out to assess the effect of atropine upon the portal system. Fig. 3 illustrates such an experiment. PAH was infused at a constant rate into one of the leg veins, and the PAH excretion by the ipsilateral kidney ranged from 7 to 10 times the excretion rate of the opposite kidney. Atropine injected intravenously in a dose of 1 mg/kg promptly reduced the relative excess of PAH excreted on the injected side.

In some hens the infusion of PAH into the vein of one leg resulted in no great excess of PAH excretion in the urine from the kidney on the infused side. The administration of atropine did not alter the ratio in these animals. It is probable that in these birds the valve was originally open and atropine was not able to relax it further.

To demonstrate that the action of atropine

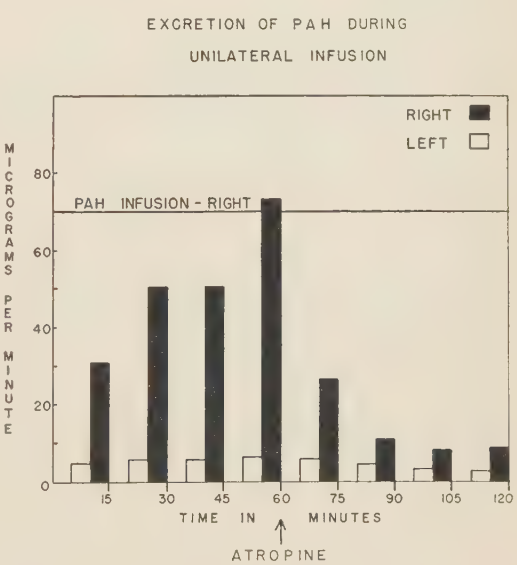


FIG. 3. Excretion of PAH from right and left kidneys when 70 μg/min. of PAH infused into right leg vein. After injection of atropine the previous abundant excretion from injected side was reduced possibly as a result of opening the venous valve.

TABLE I. Mean Values of S/M Ratios with Standard Error for PAH Uptake in Chicken Kidney Slices.

Exp.	Control	Atropine	Acetate	Atropine plus acetate
A	14.57 ± .639	12.88 ± .605*	19.45 ± .911	20.05 ± .684
B	15.32 ± .504	11.87 ± 1.054†	15.36 ± .587	16.46 ± 1.09

* 1:200000. † 1:100000. Acetate = 150 mg % final concentration.

must be due to relaxation of the venous sphincter rather than any direct inhibition of the tubular secretory mechanism, PAH uptake by chicken kidney slices was determined with and without added atropine. As shown in Table I, the PAH uptake by slices of chicken kidney in the presence of atropine was not significantly different from control slices. Table I shows the comparison of slice/medium wet weight ratios of chicken kidney tissue PAH uptake in controls with and without added acetate. Five observations were made for each value recorded. These results would indicate that the atropine-induced changes in the PAH excretion of the intact animal were not due to an effect on cellular PAH transport, but were more likely due to inhibition of the tone of the renal portal valve, permitting shunting of iliac venous blood directly into the renal vein.

Discussion. The experiments herein described suggest that the renal portal system of the chicken is not obligatory, as is the hepatic portal circulation, but is under the control of the autonomic nervous system.

Judging from the *in vitro* response of the renal portal venous valve to acetylcholine, atropine, and epinephrine, and from the *in vivo* response of the valve to atropine it could be concluded that this smooth muscle structure is contracted by a cholinergic mechanism and relaxed by adrenergic discharge. If one assumes that the response to emergency situations leads to sympathetic discharge in birds as in mammals, it follows that under conditions when muscular activity of the lower extremity is high, the renal portal shunt would remain open, permitting unimpeded venous return from the leg and reducing the volume load on the kidney. Under conditions in which leg blood flow would be expected to be minimal the valve would be closed and much of the iliac vein blood would traverse the renal parenchyma, increasing thereby the capacity of the tubular secretory and reabsorptive mechanisms.

It is of interest that this represents the only intraluminal vein valve which is known to contain smooth muscle. Perhaps the hepatic sphincter or Sperre valve, as it is known in the dog, is similar in function as are the smooth muscle bands in the hepatic veins of the seal and raccoon(5,6). The hepatic sphincter is contracted by histamine and relaxed by epinephrine. However, these structures are described as smooth muscle thickenings of the venous wall whereas the avian valve is a distinct conical smooth muscle structure affixed to the inner surface of the vein wall.

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Effect of Adrenalectomy on Tuberculin Reaction in Mice.* (20842)

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Both delayed-type bacterial allergy and acquired immunity are features of established infections in most mammalian species. The relation between these processes is difficult to assess because of complex problems which arise in setting up adequate experimental models for their study. The natural tuberculin anergy of the mouse(1), a species which may show both high and low degrees of immunity to experimental tuberculosis(2), is an exception of some interest. Tuberculin anergy in the sensitized host, although unusual, also occurs, for example during cortisone(3) and ACTH(4) treatment and in the course of some disease states(5). Since small mammals like the mouse have proportionally larger adrenals than those of heavier animals(6), it seemed possible that adrenalectomy might reverse the tuberculin anergy in mice. The present report describes the results of this procedure on mice immunized with either heat-killed tubercle bacilli in mineral oil or with active BCG infections.

Materials and methods. Tubercle bacilli. A culture of H37Rv grown on mass culture media(7) was killed at 115° for 20 minutes, washed by centrifugation from 3 changes of distilled water, and lyophilized. Suspensions of these dried organisms 10 mg/ml were prepared in light mineral oil. Turbid 8-day cultures of strains of BCG Tice and BCG Phipps maintained in Dubos liquid media(7) were used to infect mice. *Mice.* Swiss albino mice 6 weeks old weighing 20 to 30 g were divided into groups as indicated in Table I. Animals were skin tested with second strength PPD (5 γ in 0.1 ml)(8) and with brucellergen(9) 0.1 ml. Intradermal injections were done by first passing a No. 27 needle through the skin to the subcutaneous space and then maneuvering the tip of the needle into the corium from below. In this way it was possible to be certain that an intracutaneous injection was given. These skin tests produced an immediate bleb which was rapidly absorbed with the first 24 hours, leaving a non-specific 2-3 mm slightly indurated area which was gone by 30 hours.

Experimental. In the first experiment (Table I) 25 mice were skin tested and found to have negative reactions at 24 and 48 hours. Animals then received subcutaneous injections of 0.1 ml of H37Rv in mineral oil at weekly intervals for 4 weeks. The mice remained healthy and showed a moderate weight gain. After one month skin tests were repeated and found to be negative at 24 and at 48 hours. The group was divided into 3 sections as indicated in Table I. Under ether anesthesia bilateral adrenalectomy was done through a midline dorso-lumbar skin and bilateral costovertebral muscle-splitting incision. Sham operations consisted of exploration of the renal fossae and identification of the adrenals without their removal. Adrenalectomized mice were maintained postoperatively on 1% NaCl in their drinking water. In the second experiment (Table I) animals were skin tested with 10 mg O.T. in 0.1 ml of 0.85% saline 48 hours before inoculation with 0.05 ml of one or the other BCG cultures into the tail vein. These skin tests were negative at 24 and 48 hours. After operation the skin test was repeated (Table I). Autopsies on animals infected with BCG revealed many acid fast organisms in impression smears of the enlarged livers and spleens.

Results. Of the 35 mice in this study immunized with living or dead tubercle bacilli one showed a significant area of erythema and induration to tuberculin at 24 hours. This was an animal immunized but not operated upon. One sham operation animal showed a significant tuberculin reaction to a larger amount of tuberculin at 48 hours. In 3 instances slight necrosis occurred at skin test sites, in each case in animals which also showed necrosis and ulceration at the site of

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TABLE I. Skin Tests in mm of Mice in Exp. 1 and 2.

Exp.	Group	No. animals	Survived operation	Tested 48 hr postop. PPD, 5 γ 24 hr	Tested 48 hr postop. PPD, 5 γ 48 hr	Tested 96 hr postop. OT, 10 mg 24 hr	Tested 96 hr postop. OT, 10 mg 48 hr
1†	Adrenalectomy	11	6	Neg.	Neg.	Neg.	Neg.
				4 × 4 mm	4 × 4 mm	3 × 3 mm	3 × 3N* mm
				Neg.	Neg.	"	2 × 2N*
				"	"	Neg.	Neg.
				"	"	3 × 3	" *
				"	"	Died	
	Sham operation	6	5	"	" *	3 × 3	Neg.
				"	" *	4 × 4	8 × 8N*
				"	"	Neg.	2 × 2
				"	"	"	Neg.
				"	"	"	"
	Control	8	8	"	"	"	"
				"	"	2 × 2	"
				10 × 10	10 × 10*	Neg.	"
				5 Neg.	5 Neg.	5 "	5 "
2	Adrenalectomy			OT, 10 mg			
	Phipps	13	10	Neg.	3 × 3 mm		
				9 "	9 Neg.		
	Tice	12	6	3 × 3 mm	1 "		
				"	"		
				4 Neg.	4 "		
	Sham operation						
	Phipps	6	5	5 "	5 "		
	Tice	4	3	1 "	1 "		
				2 × 2	1 "		
				"	Died		

* Animals having ulceration at sites of H37Rv immunization at time skin test read
N Necrosis of 1-2 mm in center of tuberculin reaction.
† Brucellergen 0.1 ml given I.D. at 48 hr and 96 hr to surviving animals caused no reactions.
No Brucellergen tests done in Exp. 2.

the H37Rv injections. Other reactions reported were interpreted to be the result of the trauma of the procedure.

Discussion. In the present study relative tuberculin anergy in mice has been observed. Since adrenal size bears a linear logarithmic relation to body mass, it was suspected that mouse anergy might be the result of relative increased adrenal activity normal for this species and comparable to that which can be induced in guinea pigs(4) and man(3) with cortisone or ACTH therapy. Were this so, adrenalectomy in the mouse should reactivate latent tuberculin allergy. This has not been found to be the case. Although it is difficult to be certain of complete removal of all adrenal tissues unless microscopic studies are done, it is likely that regeneration of adrenal rests would not be factors within the first few days after adrenalectomy. It was within this interval that the several skin testing procedures, including one with whole dead tubercle bacilli, were negative.

Mouse leucocyte responses, as well as dermal reactions, to tuberculin differ from those of man and of guinea pig. Peripheral blood and splenic lymphocytes from tuberculous mice are lysed by tuberculin; polymorphonuclear leucocytes are not injured by tuberculin(10). In man(11) and the guinea pig(10) possessing tuberculin hypersensitivity both lymphocytes and neutrophiles may be lysed *in vitro* by tuberculin. It is possible that the failure of tuberculin to injure mouse polymorphonuclear cells in the sensitized host is related to the anergy which this species shows to tuberculin.

Summary. 1. Albino Swiss mice immunized with heat-killed H37Rv tubercle bacilli in mineral oil or with active BCG infections are

relatively anergic to PPD and O.T. used for skin tests. 2. Bilateral adrenalectomy does not reverse mouse tuberculin anergy.

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Propagation of Infectious Canine Hepatitis Virus in Tissue Culture. (20843)

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The successful propagation of poliomyelitis virus in tissue culture, recently reviewed by Weller(1), has led several investigators to apply the roller tube method to the study of other viruses. Thus, members of the Coxsackie group were also found capable of multiplication in monkey tissue cultures by Riordan and others(2), who observed a cytopathogenic effect similar to that obtained with poliomyelitis virus. Similarly, using a stable strain of human epithelial cell, Syverton and Scherer(3) reported the serial growth of poliomyelitis virus with cytopathogenic effects, as well as that of a variety of other viral agents.

The purpose of this communication is to describe the successful use of the roller tube method for the propagation of infectious canine hepatitis virus in dog kidney tissue cultures.

Materials and methods. Virus. The virus employed in this study had been used for the experimental infection of puppies, as reported in previous publications(4,5), and its origin described(5). *Tissue culture method.* Roller tube cultures of monkey, rabbit and dog kidney and of whole chick embryo in 1 ml of synthetic medium No. 199(6) containing trypsin inhibitor, were incubated at 37°C at 8 to 10 rph. The kidney tissues were grown for from 4 to 5 days and the chick embryo tissue for 2 to 3 days. The medium was renewed before infection, and no further changes were made thereafter. The inoculum consisted of 0.1 ml of 10% dog liver suspension, or infected tissue culture fluid. Infected and control tubes were observed daily for not more than 7 days. When cytopathogenic effects occurred, the infected tissue fragments and fluid were removed by scraping and suction to a Tenbroek grinder. The culture suspension thus obtained was available for either subculture or for storage in the dry ice chest. *Qualitative neutralization test.* This was done by mixing equal volumes of undiluted harvest fluids and undiluted fox immune serum previously inactivated at 56°C for 30 minutes. Controls were similarly made by mixing equal volumes of harvest fluid with either inactivated normal dog serum or inactivated ICH fox immune serum. After further incubation for one hour at 37°C, each mixture was injected in 0.1 ml amounts into groups of 3 or 4 dog kidney culture tubes. These were observed daily until complete cytopathogenic effect had taken place in the normal dog serum control tubes.

ICH fox immune serum. The standard serum was prepared in foxes as follows: seven

FIG. 1. Dog kidney tissue culture showing cytopathogenic effect following infection with ICH virus. × 175.

ranch-bred foxes were given repeated injections of a 20% dog liver suspension over a period of 3 months. They were bled out 14 days after the last injection. Their blood was pooled, the serum separated and stored in 25 ml amounts in rubber stoppered glass vials in the electric freezer. Repeated titrations of this serum pool by the complement-fixation method with infected and normal dog liver antigens consistently gave titers between 1:128 and 1:256 with the former, and no fixation whatsoever with the latter. *Complement-fixation test.* The technic used was essentially the Kolmer-Boerner test(7), which utilizes overnight icebox incubation. Infected tissue culture fluids were diluted in normal

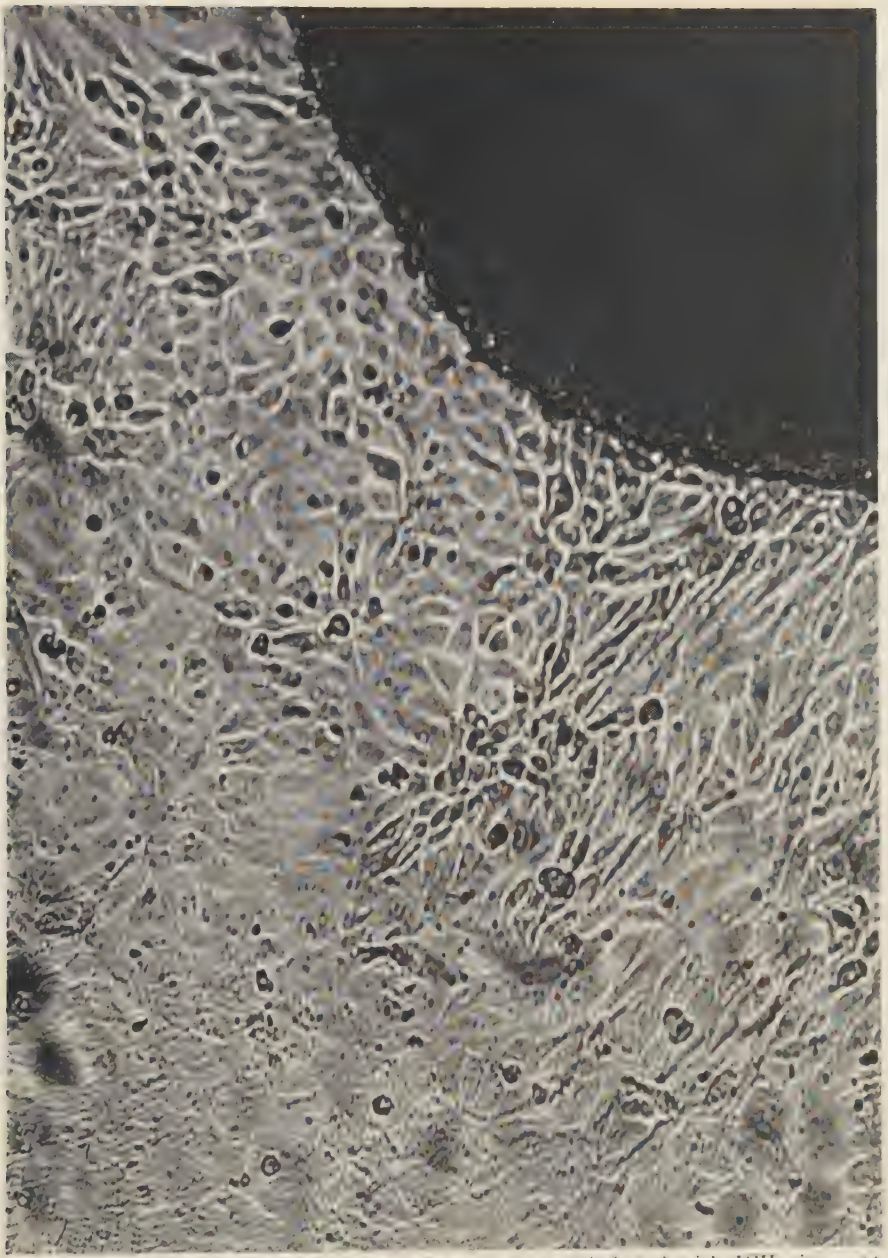

FIG. 2. Epithelial cell growth of monkey kidney tissue not infected with ICH virus. × 175.

saline in duplicate serial 2-fold dilution lines, from 1:4 to 1:128, and from 1:4 to 1:16. Addition of 8 units of immune serum to the first line of tubes and of a corresponding dilution of normal dog serum to the second followed. After distribution of 2 full units of complement, incubation was carried out at 4°C for about 18 hours. Two per cent sheep cells and 2 units of hemolysin were then added, and reading was made after further incubation for 15 minutes at 37°C.

Experimental. Serial passage of infected dog liver in monkey, rabbit and dog kidney cultures. The original inoculum of ICH virus,

which consisted of a 1:100 dilution of infected dog liver in glucosol solution(8) containing 50 units of penicillin and 50 mg of streptomycin per 1 ml, was introduced simultaneously into several tubes of monkey, rabbit or dog kidney tissue culture. Although no changes occurred in the monkey or rabbit kidney culture tubes, the fluids were harvested at the end of 7 days' incubation and those of each species pooled separately. In the dog kidney culture tubes a distinct cytopathogenic alteration in all tubes began on the fifth day following inoculation (Fig. 1), whereas control tubes remained normal throughout (Fig. 2). The infected dog kidney tubes were harvested and pooled on the sixth day. Second and third transfers were made with the 3 pools of fluid in homologous tissue cultures. Again no cytologic alterations were observed in either the monkey or the rabbit kidney series during the 7 days' incubation. However, beginning with the second passage in dog kidney culture, the cytopathogenic effect became evident on the second day and was complete on the fourth, when fluids were collected and pooled. Since the virus failed to induce any demonstrable change in monkey and rabbit kidney cultures for 3 consecutive transfers, the 2 series of passages were discontinued. However, 18 consecutive passages in the dog kidney culture series gave identical results: cytopathogenic effect started on the second day and was complete on the fourth, when fluids were routinely harvested.

Serial passage of dog kidney culture virus in rabbit kidney and whole chick embryo cultures. Passage of ICH virus in rabbit kidney cultures was attempted again, starting with harvest No. 2 of dog kidney culture. Seven consecutive transfers were made, each being harvested 7 days following inoculation. No cytopathogenic effect was observed in all 7 transfers, but assay for presence of virus which was made in dog kidney cultures at each passage level gave negative results. Passages in rabbit kidney cultures were again discontinued. A similar series was also made in whole chick embryo cultures with the sixth dog kidney passage harvest. Observation of the 6 serial transfers made in this tissue were impossible to interpret. The fibroblastic growth degenerated at the same rate in both infected and control culture tubes and masses of rounded cells began to appear on the third to fourth day. However, subcultures in dog kidney tissue at each level revealed the presence of virus in the second and third passages, but not in the fourth, fifth and sixth.

Titration of dog kidney culture virus. This was done on 4 occasions, with dog kidney harvests No. 4, 5, 10 and 15. Serial 10-fold dilutions were made with each in glucosol solution and inoculated into groups of 3 or 4 dog kidney culture tubes per dilution. Observation for 7 days revealed passage No. 4 to be completely cytopathogenic through the $10^{-4.0}$ dilution and only partially so at the 10^{-5}. The cytopathogenic effect of passages 5, 10 and 15 was complete through the 10^{-5} dilution and partial at $10^{-6.0}$. In the 4 passage levels, a gradual delay of the cytopathogenic effect was observed from the $10^{-3.0}$ dilution upward.

Comparison of ICH virus growth in normal and immune dog kidney cultures. Unless they are bred and raised in strict isolation, it is a practical impossibility to trace adequately the past infection history of most puppies or dogs acquired for experimental purposes. It seemed essential, therefore, to determine whether kidney tissue from animals immune to ICH would be unfit for the propagation of the virus. Two pups, one 5 months old and susceptible to hepatitis, and the other 3 months of age and immune following recovery from experimental infection, were sacrificed on the same day. Tissue culture tubes were separately prepared with kidney tissue from each animal. Groups of tubes from each dog were inoculated with the sixth dog kidney culture harvest in dilutions ranging from undiluted to $10^{-3.0}$. Daily observation of the tubes showed no difference in the time of appearance of the cytopathogenic effect in tissues of either animal. Following this experiment, kidney tissue from immune animals has been routinely used with satisfactory results.

Identification of Dog Kidney Culture Virus. Complement-fixation test. This test was performed on 14 consecutive harvests of the virus in dog kidney cultures. The fluids were titrated as antigens in the presence of ICH im-

TABLE I. Antigenic Complement Fixing Titers of 14 Consecutive Passages of ICH Virus in Dog Kidney Cultures, in the Presence of ICH Immune Fox Serum.

Passage No.	C.F. titer	Passage No.	C.F. titer
1	16*	8	32
2	"	9	16
3	"	10	"
4	"	11	32
5	"	12	"
6	"	13	"
7	32	14	"

* Reciprocal of highest dilution of infected fluid giving complete prevention of sheep cell hemolysis.

mune fox serum, as described under *Methods*. Results are summarized in Table I, where antigenic titers are expressed as the reciprocal of the highest dilution of fluid which completely prevented the hemolysis of sheep cells. Appreciable titers were obtained from the very first passage of the virus. A further 2-fold titer increase became evident on the 7th and 8th passages, and was uniformly obtained from the 11th to the 14th. No fixation whatsoever was obtained when the low dilutions of all fluids were tested in the presence of normal dog serum.

Neutralization test. Qualitative neutralization tests were made with harvests No. 3, 8, 9 and 13. Invariably, complete cytopathogenic effect was observed with the normal dog serum mixture by the end of the fourth day of incubation, whereas no changes whatever took place within 7 days with either of the immune fox serum mixtures.

Titration of neutralizing antibodies in ICH fox immune serum. Although the complement-fixing titer of the immune fox serum was known through repeated measurements, its neutralizing antibody content remained unknown since the only available procedure would have required a relatively large number of susceptible puppies. Those engaged in canine virus research are familiar with the difficulties involved in securing susceptible puppies for experimental purposes and in protecting them against natural infection with the agent under study. The ability of ICH virus to cause a demonstrable cytopathogenic effect in tissue culture afforded for the first time a practical method for the measurement of neutralizing antibodies. At first, the inactivated immune fox serum was diluted in glucosol in 4-fold steps, ranging from 1:4 to 1:1024. To each serum dilution an equal volume of a 1:500 dilution of the fifth harvest fluid was added, bringing the serum dilution range to 1:8 to 1:2048, and giving a final harvest fluid dilution of $10^{-3.0}$. As determined by a previous titration, the virus content at this dilution falls between 100 and 1,000 tissue culture infective doses. A mixture of equal volumes of inactivated, undiluted dog serum and 1:500 harvest fluid dilution served as control. All mixtures were incubated for one hour at 37°C, and each inoculated into 3 tissue culture tubes. Whereas the control mixture showed cytopathogenic effect starting on the third day and complete on the fifth, all immune serum-virus mixtures had no effect on the tissue culture growth in 5 days.

To determine the serum-antibody endpoint, a second test was made, identical to the first except for the serum dilution range, which extended in 2-fold steps from 1:512 to 1:8192. This time, complete protection of the tissue growth was observed through the 1:4096 dilution, while a cytopathogenic effect similar to that of the control mixture occurred at the 1:8192 dilution.

Discussion. Since the identification of ICH as a disease entity of the dog by Rubarth(9), a number of investigators have attempted the propagation of the virus outside its natural host range. Some successful efforts have been reported which, however, still remain unconfirmed. Thus, Miles and coworkers(10) passed the virus serially in the chick embryo for 12 transfers, and reproduced a mild case of the disease with the last chick embryo passage in one out of two dogs. Goret and others(11), claimed the probable transmission of dog liver virus to the ferret through nine passages. The disease induced in the ferret resembled distemper more and more with each passage, and became indistinguishable from it after a limited number of transfers. Later, Lucam and Goret(12) reported the serial propagation of the ferret-adapted agent in the embryonated hen's egg which resulted in a rapidly decreasing virulence for the ferret. Goret and others(13) reported also that the

FIG. 3. Experimental ferret showing opacification of right eye 2 to 3 wk after inj. of infected dog liver. Note clarity of left eye which received normal dog liver at same time.

experimental disease was reproducible only in ferrets of French origin, and not in those obtained from England. Furthermore, ferrets of French origin seemed to be susceptible only during a definite period of the year. Finally, Goret *et al.*(14) noted a febrile response in rabbits inoculated with infected dog liver, obtaining this response through 15 serial rabbit transfers. Except for a brief mention of the successful reproduction of the disease in susceptible puppies with the ferret-propagated virus(13), there were no attempts to demonstrate the identity of the chick embryo grown virus or of the agent responsible for the febrile response in rabbits either by serological means or by direct puppy inoculation.

Two of the present authors(15) tried repeatedly to propagate ICH virus in mice, hamsters, rabbits, distemper-immune ferrets, or chick embryos, using various routes of inoculation, with negative results. The susceptibility of the distemper-immune ferret was limited to the extent that, when infected dog liver suspension was injected intraocularly, a frank corneal opacification ensued (Fig. 3). This lasted for several months, with gradual clearing and return to normal. No such opacification occurred when a normal dog liver suspension was introduced into the second eye of the same animal. At no time were symptoms of generalized infection noted, even following simultaneous inoculations of large doses of infected dog liver suspension by the intracerebral and intraperitoneal routes. However, specific ICH complement-fixing antibodies were demonstrated in the sera of ferrets one and one-half and three months following inoculation of infected dog tissue by any route.

Although the present cultivation of ICH virus has not removed the agent from its normally susceptible host range, it represents a great advantage to the research worker over the use of live puppies. It constitutes a relatively simple and reliable method of virus assay which is being used advantageously in this laboratory in further attempts to adapt the virus to the chick embryo and other laboratory animals. It is also useful for obtaining accurate measurements of serum antibodies by the neutralization method.

A first practical application of the tissue culture virus has been the preparation of complement-fixing antigen by the present authors(15). Unlike the poliomyelitis antigen produced by a similar method by Svedmyr and others(16), which required a concentration of up to 600 x for use in the test, freshly harvested ICH-infected tissue culture fluids have titers high enough for use without concentration.

Of the two types of cells produced by dog kidney tissue—fibroblasts and epithelial cells—only the latter underwent cytopathogenic degeneration following virus infection. In cultures where both fibroblasts and epithelial cells grew side by side, only the epithelial cells were affected, whereas the neighboring fibroblasts retained their normal morphology. It was also observed that fibroblastic growth was more abundant with kidney tissue from younger than with that from older dogs. In one instance when embryonic dog kidney was used, almost a pure fibroblast culture was obtained with only rare epithelial cells. These embryonic cultures were found unsuitable for use in this study, since no cytopathogenic

effect became apparent even after seven days' incubation. However, by contrast, almost pure cultures of epithelial cells were obtained when the kidney tissue of puppies six months of age or older was used. As best as could be determined, satisfactory cultures were regularly produced with kidney tissue from pups at least 3 months old.

The effect of the tissue culture virus on susceptible puppies is being studied at the present time, and will be reported later.

Summary. The serial propagation of ICH virus by the roller tube method in dog kidney tissue culture through 15 consecutive passages is reported. The growth of the virus under these conditions causes a specific cytopathogenic degeneration of the tissue growth, similar to that reported for poliomyelitis virus in the presence of human or monkey tissues. Identification of the tissue culture virus was made by both the complement-fixation and serum neutralization methods using a known infectious canine hepatitis fox immune serum.

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Effect of 1,1-Dimethyl-4-Phenylpiperazinium Iodide on Peristaltic Reflexes of Isolated Guinea Pig Ileum. (20844)

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1,1-Dimethyl-4-phenylpiperazinium iodide (DMPP) was shown to be a potent stimulant of the autonomic ganglia(1). The question has been raised as to whether or not this compound produces paralysis of the ganglia at high concentrations as does nicotine. The inquiry is of theoretical interest in studying the chemical transmission of nerve impulses, since the ganglionic stimulating and paralyzing actions of nicotine may be explained by depolarization and overdepolarization at the synapses induced by low and high concentrations respectively of the liberated acetylcholine(2). In our experiments on pentobarbitalized dogs, no difference in hypertensive response was noticed upon repeated intravenous injections of submaximal doses of DMPP (20-30 μg/kg). However, by previous administration of 0.75 mg/kg of N-(2-bromoethyl)-N-ethylnaphthalene methylamine.HBr (SY 28, an adrenergic blocking agent) in order to suppress partially the hypertensive effect of DMPP, there was produced some diminution in hypertensive response to the second injection of 0.1 mg/kg of DMPP 5 minutes later(3). These results seem to indi-

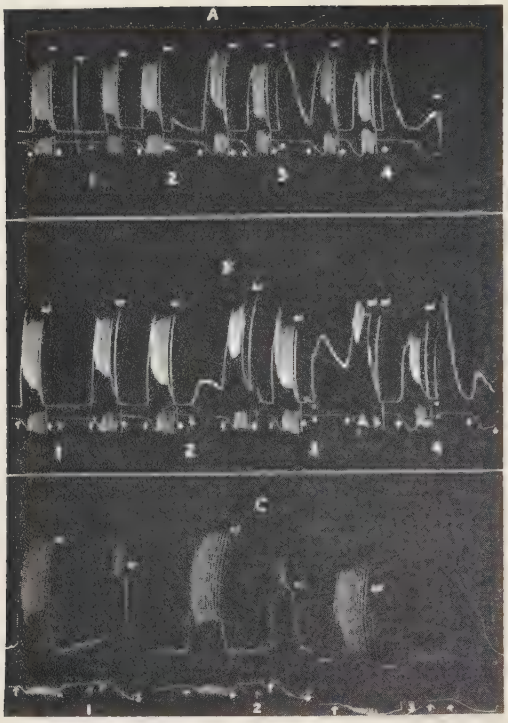

FIG. 1. Comparison of effects of DMPP, nicotine, and hexamethonium on peristaltic reflexes of an isolated guinea pig's ileum. Upper tracing—contractions of longitudinal musculature. Lower tracing—contractions of circular musculature; ω—change to fresh Tyrode solution. Peristalsis was induced by the increase of hydrostatic pressure in the lumen (in cm) for 1 or 2 min. as indicated by the arrow. The test compound in milligram quantities was added and left in the bath for 3 min. prior to the induction of peristaltic reflexes. In graphs A and B, DMPP and nicotine are shown to produce a spasmodic effect on the quiescent intestine; they increase the peristaltic reflex activity at low concentrations (0.1-0.2 mg) but suppress it at higher concentrations (0.4 mg). No stimulating effect is evident with "C_6" at any concentration (Graph C).

The concentrations of the added compounds are indicated by numbers as follows:

(A) 1, 2, 3, 4 = 0.05, 0.10, 0.20, 0.40 mg DMPP respectively.

(B) 1, 2, 3, 4 = 0.05, 0.10, 0.20, 0.40 mg nicotine respectively.

(C) 1, 2, 3 = 0.4, 0.5, 0.6 mg "C_6" respectively.

cate that DMPP is either rapidly eliminated from the site of action or a high concentration of DMPP is required to paralyze the ganglion. In view of such possibilities, any paralyzing action of DMPP appears to be best studied in an isolated organ whereby a constant and high concentration of it may be maintained.

The effect of DMPP on the peristaltic reflexes of an isolated guinea pig's ileum was then investigated. The results obtained for DMPP together with those for nicotine, hexamethonium ("C_6"), d-tubocurarine, and physostigmine are given in this report.

Experimental. For recording the peristaltic reflexes of the intestine, the method of Trendelenburg was employed(4). A clean strip of guinea pig ileum about 6 cm long was placed in a 100 ml muscle chamber with Tyrode solution at 37°C. The upper end of the intestine was closed and tied to a muscle lever to record contractions of the longitudinal muscle. The lower end was connected with

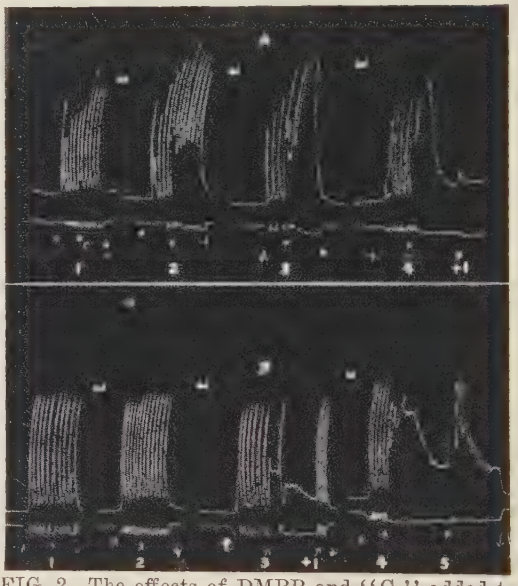

FIG. 2. The effects of DMPP and "C_6" added to the bath during peristalsis. These recordings are similar to those in Fig. 1. Peristaltic activity was increased by DMPP at low concentrations (0.1-0.2 mg), but suppressed at higher concentrations (0.4 mg). Once suppressed, there was no resumption of peristalsis by further increase of pressure (3 cm to 4 cm). Peristaltic activity was suppressed by "C_6" at all concentrations (0.4-0.6 mm); normal peristalsis was reinduced by the increase of pressure (3 cm to 4 cm) and by DMPP (0.4 mg).

The concentrations of the added compounds are shown by numbers as follows:

(A) 1, 2, 3, 4 = 0.10, 0.20, 0.30, 0.40 mg DMPP respectively.

(B) 1, 2, 3, 4 = 0.20, 0.30, 0.40, 0.60 mg "C_6" respectively.

5 = 0.4 mg DMPP.

A further increase of pressure in the lumen is indicated by the "+1" sign.

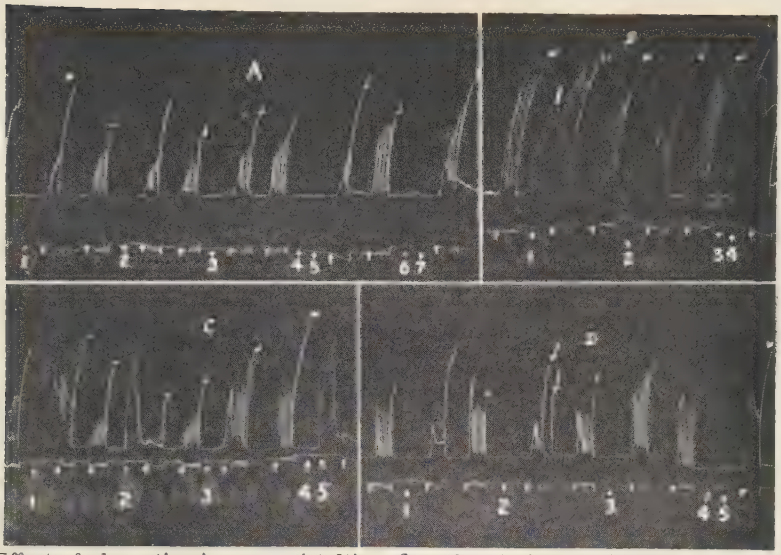

FIG. 3. Effect of physostigmine on peristaltic reflex of an isolated guinea pig ileum in response to d-tubocurarine, nicotine, DMPP, or "C_6." The recordings were made as were those in Fig. 1. The time intervals in min. between the addition of each substance and the induction of peristaltic reflexes, and the duration of peristalsis (1 min.), are given below. As shown by the last part of each graph, physostigmine (5 and 10 μg) antagonized only the paralyzing effect of d-tubocurarine (1 mg, graph A), but did not affect that of nicotine (0.25 mg, graph B), DMPP (0.4 mg, graph C) or "C_6" (0.15 mg, graph D).

The concentrations of the added compounds are indicated by numbers as follows:

(A) 1, 2 = 1.0, 0.5 mg d-tubocurarine respectively; 2 and 4 = 2.5 μg physostigmine; 5 = 1 mg d-tubocurarine; 6 = 5.0 μg physostigmine; 7 = 1 mg d-tubocurarine.
(B) 1, 2 = 0.10, 0.25 mg nicotine respectively; 3 = 5 μg physostigmine; 4 = 0.25 mg nicotine.
(C) 1, 2 = 0.30, 0.40 mg DMPP respectively; 3 and 4 = 10 μg physostigmine; 5 = 0.4 mg DMPP.
(D) 1, 2 = 0.20, 0.10 mg "C_6" respectively, 3 and 4 = 5 μg physostigmine; 5 = 0.15 mg "C_6."

tubing to the base outlet of a pressure bottle containing Tyrode solution. Through the top of the bottle, the changes in volume of the intestine were recorded with a tambour. The bottle was set on a stand with adjustable knobs so that the pressure in the ileum could be raised gradually at a constant rate. The intestine was allowed to stand in the muscle bath for ½-1 hour before experimentation. The minimal pressure required to induce a uniform peristalsis was then determined by raising the pressure bottle at 0.5 mm per second. The peristaltic contractions were recorded graphically at this pressure for one or 2 minutes. A consistent peristaltic reflex to the same increase of pressure at 5-minute intervals was taken as a control response. A test compound was added to the bath one or 3 minutes before the pressure in the ileum was raised to the control level. The strip was washed twice after each dose, and allowed to recover at least 5 minutes between each determination.

Results. In Fig. 1 are presented the kymographic recordings of peristalsis of isolated ilea under the influence of DMPP, nicotine tartrate, and "C_6". The record shows also the spasmodic effect of DMPP and nicotine on the gut at the quiescent stage as they were added to the bath 3 minutes before the pressure in the lumen was increased. The spasmodic effect of DMPP, as shown previously, may be blocked by "C_6" but not by the antimuscarinic action of atropine(1). It is seen on these graphs that DMPP like nicotine augmented the peristaltic reflex of the ileum at low concentrations (0.1-0.2 mg/100 cc) by an increase in the force of contraction with less relaxation of both the longitudinal and the circular musculature. At higher concentrations, however, DMPP prevented, as nicotine did, the induction of reflex peristalsis by pres-

sure. On the other hand, a pure blocking effect on peristalsis is indicated by "C_6" at all concentrations.

Results of a similar nature are shown in Fig. 2, obtained in experiments by adding DMPP or "C_6" during peristalsis. These graphs are given to show particularly the return of peristalsis, which had been suppressed with "C_6", by a further increase of pressure in the gut or by DMPP. When once suppressed by DMPP, on the other hand, no resumption of peristalsis occurred by the further increase of pressure.

In view of the antagonistic action of physostigmine toward d-tubocurarine at the neuromuscular junction, the influence of physostigmine upon the suppressive effect of DMPP, nicotine, "C_6" and d-tubocurarine on peristaltic reflexes was investigated. As shown by the recordings in Fig. 3, physostigmine antagonized the blocking effect of d-tubocurarine on peristaltic reflexes but did not affect that of DMPP, nicotine, or "C_6". It suggests the possibility that the latter agents and d-tubocurarine inhibit the ganglionic response of the ileum to increasing pressure in the lumen by different mechanisms. This awaits further investigation.

Summary. DMPP has been shown, like nicotine, to augment the peristaltic reflex of an isolated ileum to increasing pressure at low concentrations but inhibits it at high concentrations. This is apparently due to its stimulating and paralyzing action on the parasympathetic ganglion at low and high concentrations, respectively. The fact that the paralyzing effect of DMPP on the ganglia was not evident on repeated administrations in animals of submaximal doses may be explained by the rapid elimination of it from the site of action. Whereas physostigmine antagonized the blocking effect of d-tubocurarine, it does not influence that of DMPP on peristaltic reflexes of the ileum.

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Influence of Incubation Time and pH Changes on Uptake of P-aminohippurate by Renal Slices. (20845)

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Cross and Taggart(1) have determined the various factors which influence the uptake of p-aminohippurate (PAH) in rabbit kidney slices. These authors have stated that the maximal uptake of PAH by renal slices occurs following a 120-minute experimental period. Furthermore, they state that pH variations between 7.0 and 7.8 have no effect on PAH slice:medium ratio (S:M Ratio) of renal slices of the rabbit. Under certain conditions the time factor as well as the pH of the buffer solution have marked effects on the S:M ratio and this report represents the data on the influence of these factors on the PAH S:M ratio determined with dog renal slices.

Methods. In some of the experiments the phosphate buffer devised by Cross and Taggart(1) was employed. In another series of experiments a bicarbonate buffer (NaCl = 0.92%, KCl = 0.042%, $CaCl_2$ = 0.024%, $NaHCO_3$ = 0.05%(2) and a 95% oxygen, 5% CO_2 gas phase were used. About 100-150 mg of kidney slices obtained from 9-18 kg dogs were placed in 20 cc beakers containing 2.8 cc of buffer and 0.0013 M PAH. Various substrates were neutralized and added in a

* Work done during tenure of a State University of New York pre-doctoral summer fellowship.

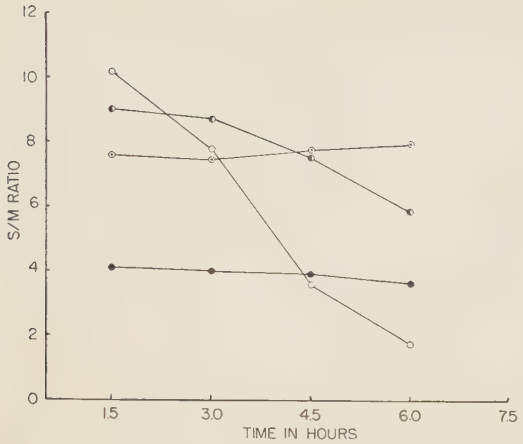

FIG. 1. Effect of incubation time on Slice:Medium Ratio of PAH. Dog renal slices, phosphate buffer according to Cross and Taggart(1). ● = no added substrate; ○ = substrate—sodium acetate .01M; Circle (shaded, left side) = substrate—sodium pyruvate .01M; ⊙ = substrate—sodium lactate (dl) .01M.

0.01 M concentration to the buffer. The beakers were shaken for varying periods in a Dubnoff metabolic shaking machine. In all these experiments the bath temperature was 25°C. Otherwise, the procedures were similar to those used by Cross and Taggart(1). Slices were prepared by means of a Stadie-Riggs tissue slicer. The S:M ratio was based on the final wet weight of the tissue.

Results. The observation was made that in the presence of sodium acetate as substrate the S:M ratio declined rather rapidly with the time of incubation. This decrease was much less marked or absent in those experiments where no substrate had been added or in the presence of sodium lactate or sodium pyruvate. Typical examples of such experiments are given in Fig. 1. The marked decline in the S:M ratio in the presence of acetate was

TABLE I. Effect of Sodium Acetate Concentration on Rate of Decline of S:M Ratio of PAH with Time. Dog renal slices, phosphate buffer according to Cross and Taggart(1). Bath temp. 25°C.

Incubation time, hr	NaAc 0.005M, S:M ratio	NaAc 0.015M, S:M ratio
1.5	9.2	8.7
3.0	6.6	6.7
4.5	4.4	4.0
6.0	2.1	1.8

TABLE II. Influence of Time on S:M Ratio and pH of the Buffer. Dog kidney slices in phosphate buffer according to Cross and Taggart(1). Bath temp. 25°C.

Incubation time, hr	No substrate (6)*		Sodium acetate .01M (5)*		Sodium pyruvate .01M (4)*		Sodium lactate .01M (4)*	
	S:M ratio	Δ pH 7.34†	S:M ratio	Δ pH 7.34†	S:M ratio	Δ pH 7.33†	S:M ratio	Δ pH 7.43†
1.5	3.99 (2.63-4.97)	.03 (-.12)	9.63 (7.03-11.95)	.40 (.35-.47)	8.53 (7.21-8.85)	.36 (.32-.42)	7.83 (5.73- 9.53)	.08 (.04-.12)
3.0	3.78 (2.93-4.44)	.11 (.07-.17)	4.90 (4.30- 5.86)	.64 (.63-.65)	7.40 (6.05-9.45)	.58 (.48-.65)	8.0 (6.0 - 9.86)	.20 (.20-.20)
4.5	3.88 (2.87-4.79)	.21 (.08-.27)	2.30 (1.41- 3.88)	.79 (.70-.85)	7.15 (5.89-8.20)	.70 (.63-.78)	8.55 (7.88-10.35)	.34 (.32-.36)
6.0	3.56 (2.47-3.76)	.19 (.09-.27)	1.47 (.75- 2.64)	.85 (.75-.95)	5.85 (3.88-7.85)	.79 (.70-.88)	8.15 (7.50- 9.20)	.45 (.32-.50)

* No. of experiments.
† Initial pH of buffer.

TABLE III. Effect of pH of Buffer Solution on PAH Uptake. Dog kidney slices in phosphate buffer(1). Incubation time 2 hr. Bath temp. 25°C.

Initial pH avg	No substrate added (4)*	Sodium acetate .01M (4)*	Sodium pyruvate .01M (3)*	Sodium lactate .01M (3)*
6.49	3.40 (2.10-4.2)	10.95 (8.65-14.5)	—	9.6 (6.3-11.5)
7.02	3.38 (2.10-4.10)	10.8 (6.56-10.80)	10.9 (7.2-13.2)	8.3 (5.6- 9.9)
7.49	3.25 (2.20-4.40)	8.75 (6.58-10.80)	9.8 (7.4-11.4)	8.0 (5.8-10.0)
8.01	2.68 (2.42-3.10)	5.76 (3.54- 8.29)	8.9 (7.2-11.2)	7.0 (4.7- 9.4)
8.52	2.1 (2.0 -2.3)	3.92 (1.4 - 6.4)	8.2 (5.4-11.7)	6.4 (4.2- 8.9)
9.02	—	1.61 (0.7 - 4.3)	6.4 (5.7- 8.4)	—

* No. of experiments.

studied further and it could be shown that neither calcium, pantothenate (0.0005 M) or α ketoglutaric acid (0.0015 M) prevented this decline. The possibility that substrate exhaustion could account for this phenomenon was considered. Since the rate of decline of the S:M ratio with time in the presence of 0.005 or 0.015 sodium acetate was the same, it is unlikely that substrate depletion was the major cause of this phenomenon (Table I).

It is apparent that the decline in the S:M ratio with time is much greater with sodium acetate than with any of the other substrates studied. The oxidation of every mole of sodium acetate would liberate one equivalent of hydroxyl ion. The determination of the pH of the buffer at the beginning and end of the observation period has shown that in the presence of substrate the pH of the phosphate buffer increased with time. In the presence of sodium acetate the increment in pH amounted to about 0.85 unit over a period of 6 hours (Table II). This change in pH could be the cause of the depressions of the S:M ratio with time. We have thus determined the effect of pH variations in the buffer solution on the S:M ratio in the absence of added substrate and in the presence of 0.01 M sodium acetate, pyruvate or lactate. Table III summarizes the data obtained and it is clear that, in the presence of acetate, an increase in pH produced a more profound depression than in the presence of the other substrates studied.

One possible reason for the decline of the S:M ratio, with acetate as buffer, could be acetylation of PAH which then would simulate a decline in the PAH S:M ratio. This was not likely since recovery of PAH in all these

TABLE IV. Effect of Incubation Time on the S:M Ratio of Acetyl p-Aminohippurate Determined with Dog Renal Slices.

Incubation time, hr	Sodium lactate .01M S:M ratio	Δ pH	Sodium acetate .01M S:M ratio	Δ pH
1.5	4.22	.14	5.87	.33
3.0	4.82	.34	4.88	.42
4.5	4.85	.29	2.4	.58
6.0	3.64	.42	1.67	.64

experiments was about 95-100%. To further exclude this possibility we have repeated these experiments with acetyl p-aminohippurate (AcPAH). Table IV shows that the S:M ratio of AcPAH declines rapidly in the presence of sodium acetate but does not do so in the presence of sodium lactate. The fact that the recovery of PAH is complete and that AcPAH behaves in a manner similar to PAH definitely eliminates acetylation of PAH as being the cause of the decline in S:M ratio when sodium acetate is the substrate.

Since the increase in pH was the likely cause of this decline in S:M ratio when sodium acetate was the substrate, we have attempted to prevent pH changes by means of a bicarbonate-CO_2 buffer system. Fig. 2 gives the average results of two such experiments and it is clear that in the presence of bicarbonate buffer the pH increase seen with phosphate buffer does not occur to the same extent and furthermore the decline in the S:M ratio is also prevented. If 100% oxygen was used in conjunction with bicarbonate buffer the pH of the buffer rapidly increased from about 7.2 to 8.25 and the S:M ratio was depressed below 1.0.

It could also be shown that in bicarbonate buffer and in the presence of sodium acetate

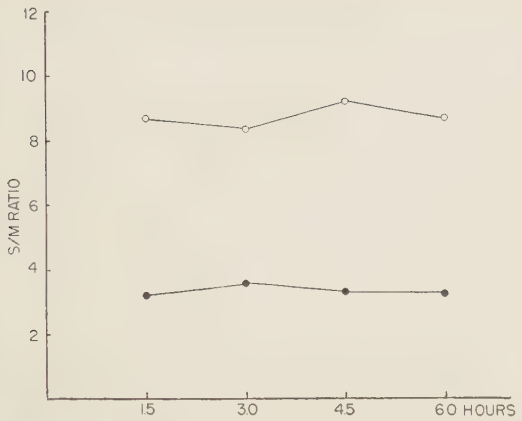

FIG. 2. Effect of incubation time on Slice:Medium Ratio of PAH in presence of bicarbonate buffer (pH 7.2). Average change in pH over a 6 hr period was 0.14 pH unit. Dog renal slices. ● = no added substrate; ○ = substrate—sodium acetate .01M.

(.01 M) the decline of the S:M ratio of AcPAH did not occur and the pH of the buffer remained relatively constant.

Discussion. The data presented have shown that the decline in the S:M ratio of PAH with time is related to the pH changes in the buffer solution occurring in the presence of the various substrates. However, this pH change is not the only factor since acetate and pyruvate behaved differently although the increment in pH change was about the same for both substrates. A possible mechanism is the higher resistance of the pyruvate system towards pH changes demonstrated in Table III. The possibility that acetylation of PAH may account for this decrease of the S:M ratio in presence of acetate as substrate was considered. Calculations of the recovery of PAH in the system did not support this hypothesis since the same amount of PAH was recovered (95-100%) in 1.5-hour experimental series as in the 6-hour series. Further evidence that acetylation of PAH is not the cause was presented in Table IV where acetyl PAH showed a decline similar to that of PAH in the presence of sodium acetate. The demonstration that in the presence of bicarbonate buffer the pH changes as well as the decline in the S:M ratio were absent strongly indicated that the main factor explaining this decline observed in phosphate buffer and sodium acetate as substrate were the pH changes. The differences in sensitivity to pH changes between lactate and pyruvate on the one hand and acetate on the other are shown in Table III. This difference in sensitivity to pH changes explains why in the presence of pyruvate and lactate the decline in the S:M ratio with time was much less than in the presence of sodium acetate as substrate. Why the pyruvate and lactate systems are more resistant than the acetate system to pH changes is not clear. It is possible that a fraction of the pyruvate and lactate may be oxidized by metabolic pathways different from those of acetate.

Summary. In the presence of sodium acetate as substrate the S:M ratio of PAH declines rapidly with time. With no added substrate and with sodium lactate or sodium pyruvate as added substrate this decline in the S:M ratio is much less, or even absent. It is shown that the increase in pH occurring in phosphate buffer is the cause of the decline of the S:M ratio.

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Non-Specific Reversal by Vitamins of Inhibition of Ethanol Oxidation by Antabuse (Tetraethylthiuramdisulfide) in the Rat. (20846)

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Acetaldehyde was found to accumulate upon administration of ethanol(1) in animals, treated with TTD (tetraethylthiuramdisulfide($[(C_2H_5)_2NCS_2]_2$). While the overall ability of the body to dissimilate acetaldehyde remains unchanged, the rate of oxidation to acetic acid is greatly retarded through the inhibitory action of TTD. The nature of this inhibition is not yet clear. The interference with the oxidation of ethanol in TTD-treated animals is coupled with toxic symptoms. According to Lecoq(2), the toxicity is due to a disturbance of pyruvate metabolism rather than to the accumulation of acetaldehyde. This conclusion is based on the observations that injection of pyruvate produced the same symptoms as ethanol in TTD-treated animals, and that the toxic effects of both pyruvate and ethanol could be reversed by administration of nicotinamide and adenine. This author found that thiamine, but not riboflavin, could also reverse the toxicity of pyruvate and ethanol in TTD-treated animals. Ascorbic acid was found to counteract the toxicity of ethanol, but not the pyruvate(3). Lecoq *et al.* also found meso-inositol, cysteine-HCl or sodium succinate to be ineffective(4). Cortisone had slight and ACTH no effect at all. The reversibility of the inhibition of acetaldehyde oxidation by TTD *in vitro* by Racker's aldehyde dehydrogenase system on additions of diphosphopyridine nucleotide, glutathione, and to a lesser extent ascorbic acid was reported by Graham(5).

Because of the lack of data concerning acetaldehyde blood level changes following vitamin treatment in TTD-ethanol-injected animals, we have explored this problem in intact rats. The damage caused to the metabolic capacity of the liver following continuous administration of TTD was also investigated. In a preliminary communication the reversal of the effect of TTD by vitamins was shown to be competitive and non-specific(6). The details of the work are presented here.

Material and methods. Male rats of the Long-Evans strain, weighing 250-300 g (unless otherwise stated) were used. They were kept on a Purina Lab Chow diet with water given *ad libitum*. The commercial tetraethylthiuramdisulfide (obtained through the courtesy of Sharples Chemical Co., Philadelphia, Pa.) was crystallized from asym. propylene glycol. On cooling, it crystallized in long needles melting at 70°. The ethanol used was distilled over sodium and kept under anhydrous condition prior to its dilution with water. The determination of the blood acetaldehyde concentration was carried out according to the spectrophotometric method of Burbridge *et al.*(7). The absorption of acetaldehyde semicarbazone was measured at 224 mμ. For analysis 2 ml blood was withdrawn with a heparinized needle from the heart of unanesthetized rats and incubated in an all glass modified Conway cell. Liver homogenates were checked for the degree of enzyme inhibition following the administration of TTD by the Thunberg methylene blue method. Changes in the methylene blue concentration were measured by the light transmission at 660 mμ in a Klett-Summerson photometer(8). The inhibition was expressed in % by the equation: % inhibition = (C-X)100/C, where X is the change in transmission of the TTD-treated and C the change in the transmission of the controls.

Results. Each of the 5 rats per group received 20 mg of TTD in one ml of asym. propylene glycol. (kept at 38°C) intraperitoneally for 2 successive days. A single dose of 40 mg killed the animals. On the second day ½-hour following the TTD injection, the vitamins were administered intraperitoneally in 1.0 ml of aqueous solution. The controls were given 1.0 ml of distilled water. One

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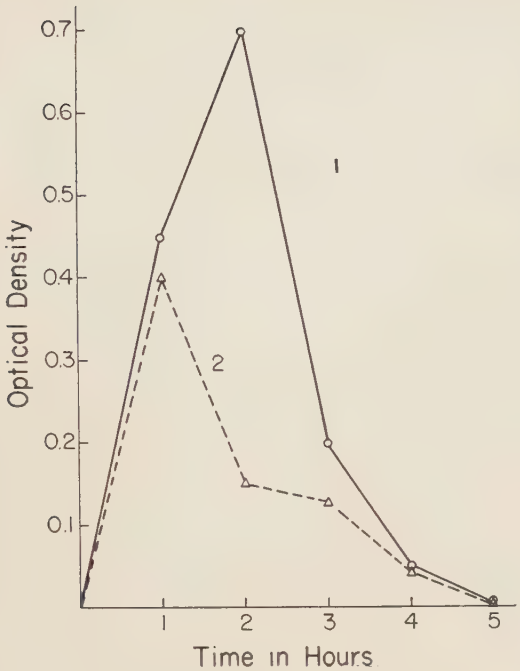

FIG. 1. Effect of nicotinamide on blood levels of acetaldehyde in antabuse-treated rats. Curve 1. Animals of group given 20 mg TTD for 2 days followed by 0.197 g ethanol. Curve 2. Same but with 20 mg nicotinamide.

half-hour later all the animals received 0.197 g of ethanol in 1.0 ml of water intraperitoneally. This concentration of ethanol was tolerated by the rats without mortality and produced measurable amounts of blood acetaldehyde within the first 15 minutes. Fig. 1, curve 1, shows the change in acetaldehyde concentration with time. The peak of acetaldehyde accumulation occurred at about 2 hours and disappeared at about 5 hours. The injection of 20 mg of nicotinamide (1.64×10^{-4} mole) decreased the blood acetaldehyde (Fig. 1, curve 2). At 1.64×10^{-5} molar level the above effect of nicotinamide is not reproducible.

The *in vivo* action of nicotinamide does not appear to be specific and is produced also by other vitamins. Table I shows the results obtained with other vitamins at 2 and 20 mg levels respectively. The lower values obtained with ascorbic acid are in agreement with the *in vitro* observations of Graham(5) that, in restoring the aldehyde dehydrogenase activity following TTD inhibition, a greater molar ratio of ascorbic acid to TTD is required than with either diphosphopyridine nucleotide or glutathione. TTD given alone(9) or with asym. propylene glycol. does not lead to a rise of the blood acetaldehyde.

The antidotal effect of nicotinamide following alcohol administration was tested in 24 male rats weighing 400-500 g in 2 experiments. The first experiment employed 8 rats and the second 16. The animals were treated with two 20 mg doses of TTD on successive days. On the second day they received 0.25-1.0 ml pure ethanol intraperitoneally followed half an hour later by 40 mg of nicotinamide in 1.0 ml of water. No reversal of either the toxic symptoms or of the aldehyde levels was observed.

In vitro experiments showed that TTD exerted a powerful inhibition on SH enzymes, *e.g.*, rhodanese(6) and succinic dehydrogenase (5). An experiment was designed to test whether TTD would damage enzymes *in vivo* upon continued administration. Fifteen male rats were injected daily with 20 mg of TTD in 1.0 ml of asym. propylene glycol. for 21 consecutive days. No mortality was observed. At the end of the period the animals were killed and homogenates of their livers were tested for impairment of their ability to reduce methylene blue in the presence of succinate, citrate and ethanol. The inhibition of the dehydrogenase activity was 38% for citrate, 60% for ethanol and 45% for succinate.

Discussion. In the above experiments it was found that about 1/10 of the amount of TTD and 1/25 of the amount of ethanol employed by Lecoq(3) caused little toxicity, but produced a significant accumulation of acetaldehyde. All the vitamins assayed could reverse the accumulation of acetaldehyde produced by TTD. Injection of chemicals like asym. propylene glycol. or cystine had no influence.

The generalized effect of TTD, on the basis of these experiments, is thought to be produced by its action on SH-groups. We also found in two experiments that 20 mg of calcium pantothenate reversed the action of TTD. The non-specific effect of the vitamins would by no means be surprising since, al-

TABLE I. Reversing Effect of Vitamins *In Vivo* on Inhibition of Acetaldehyde Oxidation by TTD.*

Group	Avg aldehyde blood levels (mg %) (a)	(b)	mml vit. required for 100% reversal of 1 mml TTD†
TTD + ethanol	1.38	1.22	—
+ nicotinamide + ethanol	1.20	.58	2.3
+ pyridoxine HCl + "	1.14	.57	1.4
+ riboflavine + "	1.24	.56	.8
+ thiamine HCl + "	1.25	.38	.6
+ folic acid + "	1.30	.60	.6
+ ascorbic acid + "	1.35	.87	3.0
Ethanol alone‡			
TTD " ‡			

* 2 hr after intraper. inj. of 0.197 g ethanol. Each figure represents 5 animals. (a) 2 mg; (b) 20 mg vit. inj.

† Calculations based on column (b).

‡ Below limit of sensitivity.

though the enzymatic processes involved in the oxidation of alcohol to acetic acid are not as yet well understood, there is every indication of the existence of several pathways of alcohol oxidation.

The amount of the vitamins required to yield reversal of TTD is shown in Table I. At present no explanation is offered for the inability of the vitamins to reverse the TTD-effect at their lower concentration.

Summary. In TTD-treated rats certain vitamins are capable of reversing the inhibition of TTD on alcohol oxidation at the acetaldehyde level when injected prior to ethanol administration. The effect of two 20 mg doses of TTD can be reversed by 20 but not by 2 mg of the vitamins. Chemicals like propylene glycol. and cystine did not produce reversal. Injection of nicotinamide into TTD-treated rats after ethanol does not relieve or reverse the toxic symptoms. There is damage to the liver of the TTD-treated rats after 21 days, as shown by its impaired ability to transfer hydrogen anaerobically to methylene blue using succinate, citrate and ethanol as substrates. The damage is thought to be produced through the affinity of TTD for the sulfhydryl sites of the enzymes.

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Preparation and Properties of a Purified Ragweed Extract.* (20847)

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The isolation and purification of the active principle of ragweed pollen has been the subject of considerable study. The earlier work has been adequately reviewed by Newell(1). More recently, separations have been made by electrophoresis(2,3), precipitation at pH 3-4(4), phosphotungstic acid precipitation (5), and adsorption on alumina gel(6). In continuation of the work of Abramson *et al.* (3), we have found that the colorless fractions prepared electrophoretically still contain considerable impurity. It was also found that pigment could be removed in a variety of ways including electrophoresis and solvent extraction. A method of removing the pigment is described which involves the extraction of the pollen grains with 90% methanol before preparing the aqueous extract in the usual way.

Materials and equipment. Giant ragweed pollen was used after being defatted with ether. Reagents were all A.C.S. or C.P. grade. The electrophoretic separation cell was similar to that described previously(3). The method of Tiselius, with a Perkin Elmer instrument (9), was employed to study a methanol purified extract by analytical electrophoresis. Absorption spectra were obtained on a Beckman D. U. Spectrophotometer. The values given for the region 2050 Å to 2200 Å are not corrected for scattered radiation(7), but the values are qualitatively comparable.

Experimental. a) *Electrophoretic separation.* Trifidin, as well as the migrating pigment, was prepared as described by Abramson *et al.*(3), and the absorption spectra of these were compared with those of the crude extracts. These are given in Fig. 1 where two main absorption peaks are observed for the crude extract at about 2600 Å and 3700 Å (Curve A). On electrophoretic separation, the curve for the "colorless" Trifidin (Curve B) still shows the marked band at 2700 Å and a flat portion at 3600 Å. The spectrum of the colored fractions which migrated away from Trifidin gives a curve (Curve C) closely following the shape of the crude extract. b) *Solvent studies.* It follows from the rationale of the electrophoretic method that the crude extract should have as low a conductance as is possible(3). It was found by conductometric measurements that extraction of pollen in the cold with 90% methanol (V/V) removed a large fraction of conducting salts from pollen. At the same time, a large amount of color was also removed. Extraction with water of the methanol-leached pollen, however, still yielded a faint yellow solution. The difference between this pigment and that extracted with 90% methanol is shown by the reaction with alkali. The color of solutions of the latter (90% methanol extracted pigment) deepened in alkali (pH 9)

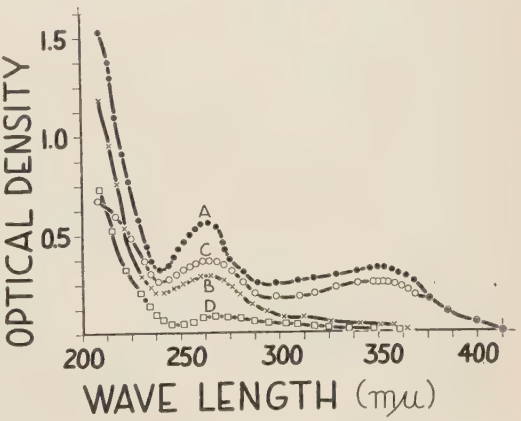

FIG. 1. Absorption spectra of electrophoretically separated pollen extracts. A: crude extract. B: Trifidin fraction. C: pigmented fraction. D: Trifidin precipitated with methanol.

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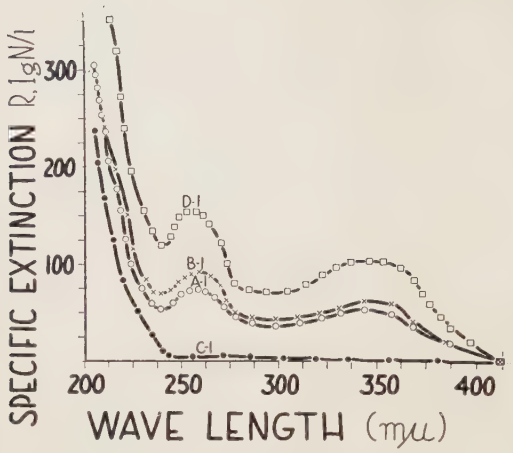

FIG. 2. Specific extinction absorption curves (k, N = 1 g/l). B-1: crude lyophilized fraction. A-1: MLL. C-1: precipitate from reprecipitation of MLL. D-1: methanol extract from reprecipitation of MLL.

and was lighter in acid (pH 2). The former pigment requires relatively stronger alkali before any change is noted and seems to be unaffected by acid. For biological testing, samples were prepared as follows: 5 g of defatted giant ragweed pollen was leached 6 times for 10 minutes in 50 ml 90% methanol. The sixth extract was essentially colorless. The air-dried pollen was then extracted with 100 cc water in the refrigerator for 48 hours. The mixture was filtered and the filtrate lyophilized. The lyophilized residue was a light yellow powder which, on dissolving in water, gave curve A-1, Fig. 2. The general shape of curve A-1 is the same as the crude lyophilized fraction (curve B-1), but the 2600 Å and 3600 Å bands were depressed. This material is called MLL (Methanol Leached Lyophilized). In general, about 100 mg of dry powder was obtained from 5 g of pollen. This powder was soluble in water and had approximately the same nitrogen content as the crude extract on a weight basis (Table I). A sample of MLL was dissolved in water and precipitated with 9 volumes of absolute methanol. The precipitate was separated by centrifugation. The supernatant gave curve D-1 and the precipitate, curve C-1, Fig. 2. The dilutions were made so that curve C-1 plus D-1 should give A-1 and this is correct within 5%. Again the alcohol solution had a predominance of the impurity with peaks at 2600 Å and 3600 Å whereas the precipitate showed neither band.

TABLE I. Analyses of Ragweed Extracts.

Preparation	Nitrogen (%) dry wt
Crude extract	4.85
MLL	5.1
MLL precipitated with methanol	6.3

Clinical study of biological activity of methanol washed, lyophilized giant ragweed pollen. This study was performed on 5 patients all of whom had a clinical history of ragweed hay fever. The patients ranged in age from 10 to 15 years. All patients were scratch-tested with alternating controls to giant ragweed pollen. Of the 5 subjects tested, 2 were found to give positive reactions. All 5 subjects were tested intradermally in triplicate with various dilutions of 1) MLL and 2) control ragweed. Carefully measured quantities of 0.03 cc were injected intradermally. The dilution range was from 0.001 (W/V), 0.001, 0.01, 0.1 and 1 mg/cc respectively.

TABLE II. Biological Tests of MLL in 5 Subjects.

mg/cc (W/V)	Methanol washed ragweed (cm)	Control ragweed (cm)
.0001	0	0
.001	.3, w†	.3, w
.01	.7, w—1 knob	.6, w—1 knob
.1*	1.4, w—2 p†	1.5, w—3 p
.0001	0	0
.001	.5, w	.4, w
.01	.8, w	.8, w
.1*	1.8, w—3 p	1.6, w
.0001	0	0
.001	0	0
.01	.6, w	.5, w
.1	.9, w	.9, w
1.0	1.2, w	1.1, w
.0001	0	0
.001	0	0
.01	0	0
.1	.3, w	.3, w
1.0	.6, w	.6, w
.0001	0	0
.001	0	0
.01	0	0
.1	0	0
1.0	.4, w	.5, w

* In these two subjects, because of the size of the 0.1 mg/cc reaction, no higher concentration was used.

† w = wheal; p = pseudopods.

Comparative dilutions of (1) and (2) were used in the testing. The results are summarized in Table II, which disclosed no difference between crude extract and MLL. A qualitative test of activity was made on the precipitate obtained by methanol precipitation and the sample was extremely active. In view of the spectroscopic results, it was concluded that the material which shows marked 2600 Å and 3600 Å bands is an inactive impurity.

Electrophoretic analysis. MLL was compared with extracts of pollen which had not been previously methanol leached. The electrophoretic analyses are illustrated in Fig. 3. The typical descending electrophoretic pattern of ragweed pollen extract, previously described(2), is shown. The figure on the right is the pattern for the aqueous extracts after treatment of the pollen with 90% methanol. The concentrations were approximately 16 mg/cc. The methanol extracted material is somewhat less concentrated. The buffer was 0.02 M with respect to phosphate and 0.15 M NaCl with a pH of 7.4. It may readily be observed that the unpigmented fraction previously described(2) is the main component and still present with an extremely low mobility. However, there is a new, essentially unpigmented fraction with a mobility of 0.3 μ per second per volt/cm which is a new component that appears after removal of the pigments with 90% methanol.

Discussion. The argument presented above that the "colorless" Trifidin forms the basis of biological activity is well borne out not only by the electrophoretic analysis, but also by the fact that the data in Table II demonstrate unequivocally that pollen extracts containing markedly reduced pigment show the same biological activity on ragweed sensitive individuals as pollen extracts containing the large variety of pigments previously reported. One of the pigments of giant ragweed pollen has been identified as isoquercitrin(8) and was found to possess no biological activity. We believe that removal of the pigments with 90% methanol brings us closer to the understanding of the basis of the biological activity. It would appear that the leaching of defatted pollen with 90% methanol may aid in obtaining sufficient material for further chemical studies of the nature of the "colorless" molecules.‡

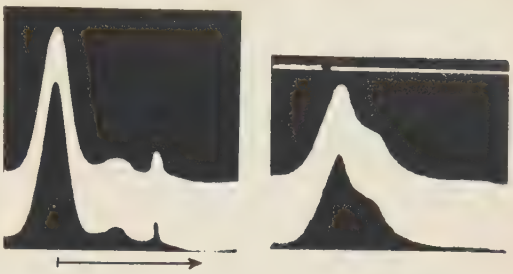

FIG. 3. The typical electrophoretic pattern of ragweed extract is shown on the left. On the right is the aqueous extract after treatment of the pollen with 90% methanol. There is a new, essentially unpigmented fraction with a mobility of 0.3 μ per sec. that forms after removal of the pigments.

Summary. Absorption spectra of ragweed pollen extracts prepared by a new electrophoretic preparative method and by extracting the pollen with methanol are reported. Two main absorption bands were observed for crude aqueous extracts at about 2600 Å and 3600 Å. The 2600 and 3600 Å bands may be correlated with an impurity. The main

‡ It is of interest to speculate on the nature of the binding forces between pigments and colorless fraction. This new essentially unpigmented fraction is active and conceivably would have the mobility of Trifidin if *all* the pigment could be removed. Since a very small amount of pigment is still present in this methanol extract an attempt was made to clear up the ambiguity related to pigment content since it was not certain what happens to the pigment. Direct observation of the electrophoresis cell did not result in any different decision because the intensity of the pigment is so low. However, it would appear that the formation of a new essentially unpigmented component following the removal of pigments supports our previous concepts(2) that the colorless material, the major practically uncharged component, represents the biologically active molecule with pigment groups lending the colorless molecules mobility but not biological activity. In other words, we believe that Trifidin is the basis of the biological activity of giant ragweed extract and that the various pigmented active molecules are in a steady state reacting with pigment groups that are readily disrupted by solvent extraction or as previously observed, electrophoresis itself. However, this must be supported by further studies with the ultracentrifuge.

components separated electrophoretically by the preparative method still showed a marked absorption band at 2600 Å, but none at 3600 Å. Ninety percent methanol leached, lyophilized ragweed pollen extracts (MLL) show a marked depression of the 2600 Å and the 3600 Å bands. Biological activity of the essentially pigment-free preparation is identical with the preparation containing all of the pigment thus confirming our previous point of view that the pigments do not contribute substantially to the biological activity. Electrophoretic analysis reveals that a new substantially colorless major component is formed, on removal of the pigment, with an electric mobility of approximately 0.3 μ per second at pH 7.4.

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Effect of 200 Roentgens Fractional Whole Body Irradiation in the Burro. (20848)

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In a previous investigation of the response of the burro to 400 r fractional whole body irradiation(1), it was observed that this species responded to such irradiation in the same manner as swine(2) and goats(3). In addition the burro demonstrated a pronounced hyperferremia one hour after the completion of the first 400 r exposure and this condition reached a peak on the sixth irradiation day. The present investigation was designed to compare the possible differences in response which might occur when the total daily dose was reduced and to compare the overall response with that seen after a single acute dose in the LD_{50} range.

Methods. Ten male burros were exposed to 200 r Co^{60} gamma ray whole body irradiation daily for 16 days using the multicurie irradiation site described by Wilding *et al.*(4). The radiation flux was approximately 48 r/hr. Control blood samples were obtained 3 times prior to irradiation and daily within one hour after the completion of each irradiation. Hemoglobin was determined colorimetrically by a modified Dupray method(5-7). Platelet counts were made with the method of Brecher and Cronkite(8) and the other hematological values were obtained by the methods of Wintrobe(9). Plasma iron values were determined by the method of Kitzes *et al.*(10) using 2 ml aliquots from plasma samples kept frozen until analyzed. Records were kept of daily body temperatures, food and water consumption and total body weight loss during the experimental period.

Results. General effects. During the first irradiation day the animals appeared normal, but were listless after 400 r and became more so with each subsequent irradiation. The

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first death occurred after 1200 r had been administered but nine of the animals received 2800 to 3200 r before dying. Irritation noticed during the initial sickness changed to a passive tolerant attitude and a pseudo-euphorism became apparent after the first irradiation week. The mean body temperature remained within normal limits (36.8-37.5°C) until 1000 r had been given, at which time a slight decrease was noted (35.7-36.8°C). A definite increase in temperature occurred and continued after 2000 r had been received (37.2-38.2°C) reaching a peak at 2600 r (37.8-39.5°C). Near death the temperatures in some animals were markedly below normal and none were recorded after the 2800 r dose. Anorexia and a reduced water intake were observed beginning upon the eighth irradiation day and continuing until exitus. The average body weight loss for the group was 2.98 kg/day. Buccal and gingival ulcerations, fecial swelling, salivation and twitching of the lips were common after the tenth irradiation day, at which time the breath of the burros was malodorous. Prolonged bleeding occurred at the veni-puncture site and blood flowed from the nares, mouth and small wounds during the 9th, 10th and 11th days. This bleeding, indicative of a blood coagulation defect, continued until exitus. Some of the animals had encephalitis-like symptoms consisting of non-directional walking and a spasmodic muscular twitching. All of them exhibited a locomotion defect characterized by a stilted walk, "popping" of the joints and "knuckling over" of other joints. Retraction of the testicles high into the scrotum and in some animals into the abdomen was noted at necropsy. There was no epilation but hyperesthesia, particularly noted by hand pressure on the back, was apparent prior to exitus. Diarrhea was not observed and equidae do not vomit. One burro (No. 301) did not confirm to the general syndrome but collapsed suddenly 43½ hours after the beginning of irradiation (400 r). Although helped to his feet, he never again was able to stand. He appeared to want to arise, eat and drink but could not. Consciousness remained until just before death, 140 hours after irradiation began (1200 r). Spasmodic muscular twitching of the

TABLE I. Effect of 200 r Fractional Gamma Ray Irradiation on Hematology and Plasma Iron of Burros. Mean values ± stand. dev.*

Constituent		Pre-irrad. control*	Irradiation days 1	2	3	4	5	6	7	8	9	10	11	12	13	14
Hemoglobin	$\bar{x}$	10.3	10.3	10.4	9.9	9.4	9.9	9.9	9.7	9.5	9.6	9.0	8.8	8.8	9.2	8.6
	±	.2	.2	.2	.3	.3	.3	.3	.1	.2	.3	.2	.2	.3	.2	.5
Hematocrit	$\bar{x}$	33.1	32.7	32.3	32.6	31.4	32.7	32.9	32.0	31.2	31.1	29.4	28.8	28.7	28.8	31.8
	±	.5	.9	.6	.1	1.0	.9	1.0	.5	.7	.9	.5	.3	.8	.7	1.0
Erythrocytes × 10^{-6}	$\bar{x}$	6.31	6.33	6.19	6.22	5.6	6.2	6.3	6.1	5.4	5.9	5.6	5.5	5.5	5.4	6.0
	±	.88	.15	.82	.10	.2	.1	.2	.1	.3	.2	.1	.1	.2	.1	.2
Leukocytes × 10^{-3}	$\bar{x}$	15.8	11.5	8.5	7.0	6.1	5.0	4.3	2.8	2.3	1.4	.8	.4	.3	.2	.1
	±	2.1	.5	.8	.4	.3	.3	.3	.3	.2	.3	.2	.1	.1	.05	.01
Platelets × 10^{-3}	$\bar{x}$	515	567	537	497	446	431	414	415	386	371	295	272.0	152	78.	34
	±	15.0	26.0	22.0	15.0	16.	16.	14.	14.	11.	9.0	22.	23.	29.	17.	9.
Plasma iron, μg %		109.7	115.9	110.3	149.8	110.6	138.1	144.9	144.7	163.2	176.9	165.2	151.9	138.8	136.6	152.4
	±	4.4	5.1	4.8	12.6	7.1	5.2	6.6	4.6	7.6	6.1	7.7	6.1	5.4	4.4	12.2

* Pre-irradiation control values represent results from 27-30 samples. All others are from 9-10 animals except last day when there were 8.

head and neck was seen from the 43rd to the 100 hour. No gross changes were observed at necropsy.

Hematological effects. The effect of the irradiation on the hematological picture is given in Table I. In general there was no great effect upon hemoglobin concentration or the number of erythrocytes until a slight decrease appeared in both on the 8th irradiation day. The values seem to indicate that the total blood loss from hemorrhage was minor up to the 9th day. The hematocrit values changed only slightly throughout the experimental period. A leukopenia began after only one exposure and became more pronounced with each subsequent exposure. Inasmuch as differential counts were not performed, no information concerning the effect of irradiation on individual leukocytic components is available. The platelets showed a slow diminution in number, characteristic of the burro, until the 11th day when half the normal number were present. Thereafter the platelets disappeared at a rapid rate reaching 6% of normal on the 14th day. The decrease in the number of platelets coincided with the appearance of the hemorrhagic symptoms. The leukocyte counts for animal No. 301 have been omitted from Table I because this burro had abnormally high counts (18,260) prior to irradiation and leukocytosis on the third day (19,900). At exitus the value was 2750.

Effect on Plasma Iron. Table I gives the daily plasma iron values for all animals. There is a considerable spread in individual animal daily iron values but the mean values indicate a significant rise within one hour after the third dose (total 600 r) had been administered, with a mean peak value after 1800 r and a slight progressive decrease until exitus. However, this latter effect did not cause the plasma iron values to approach the mean control values. The majority of animals showed significantly elevated plasma iron values prior to death.

Discussion. The overall response of the burro to 200 r daily exposure to Co^{60} gamma ray whole body irradiation was similar to that observed with both 400 r fractional(1) and single LD_{50} acute whole body irradiation(11). However, certain differences between the responses to 200 r and 400 r were apparent. The former animals had a hyperthermia and bleeding syndrome, whereas the latter ones did not. The 200 r burros showed a progressive decrease in hemoglobin concentration, erythrocyte count and hematocrit value while the 400 r animals showed no effect on the first two values, and an increase in the last. This indicates a progressive anemia and hemodilution in the 200 r group and a hemoconcentration in the 400 r group. As would be expected, the leukocyte counts indicate a slower rate of destruction from 200 r than from 400 r. The response of the platelets to both amounts of radiation was the same at similar time intervals but the 400 r animals did not live long enough to show the pronounced decrease seen in both the 200 r and the acute LD_{50} series on the 12th day. The normal plasma iron values for the 200 r series were higher than in the 400 r series but the overall response was the same and both groups reached a peak after 1800 r had been administered. The significance of this increase in plasma iron is difficult to interpret because complete information is lacking regarding its source and there is no good correlation between its presence and lethality. However, increased plasma iron has been observed in irradiated rats(12), dogs(13), burros(1) and rabbits(14), and part of this increase in circulating iron has been shown to be ferritin liberated from the liver(15,16).

Summary. A study has been made of the response of the burro to 200 r fractional Co^{60} gamma ray whole body irradiation. The response is similar to but slower than that observed with a daily dose of 400 r. The response of the platelets to irradiation with 200 r daily corresponded most closely with that seen when an acute LD_{50} dose had been given and correlated with the bleeding tendency which developed on the ninth irradiation day. A significant hyperferremia was observed in the burro one hour after receiving a total dose of 600 r but the maximum response was not obtained until 1800 r had been administered. Most animals had significantly elevated plasma iron values prior to exitus.

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Inhibition of Brain Respiration *in vitro* by Bilirubin. Reversal of Inhibition By Various Means.*† (20849)

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Kernicterus is the yellow staining of areas of gray matter found at autopsy in infants dying of erythroblastosis fetalis(1). The prevention of kernicterus and its concomitant nerve cell damage by replacement transfusion therapy provides the chief justification for that procedure(2). Other conditions than hemolytic anemia affecting the newborn, and associated with jaundice, may also be complicated by kernicterus(3,4). Although the pigment as it is found within nerve cells has been identified as mesobilirubin, the toxic agent or agents remain unknown(5). The present research was undertaken to see if bilirubin itself could be a contributing factor to the death of nerve cells. Although clinical experience indicates that jaundice is well tolerated by adults, high serum levels of indirect bilirubin in neonatal life are associated with a high death rate stemming apparently from kernicterus(6), and follow-up studies of infants recovered from erythroblastosis show an association between severe jaundice and a later impairment of intelligence(7).

Material and methods. Rats weighing from 25 to 75 g were decapitated, their cerebral hemispheres removed and at once chilled and chopped with scissors into pieces less than approximately 2 mm in their largest diameter. From 200 to 300 mg of chopped brain were placed in each of 4 to 6 Warburg flasks, the air replaced with oxygen, and oxygen uptake at 24.6°C recorded for the first 40 to 50 minutes after temperature stabilization. The fluid bathing the brain consisted of an electrolyte solution either as such for controls or with bilirubin and other test substances dissolved in it. The composition of the fluid, before the addition of bilirubin and adjustment of pH was as follows: NaCl .126, KCl .0025, Na_2CO_3 .082, Buffer .008 molar (Tris-(hydroxymethyl)Aminomethane). After the test substances were dissolved, the control and

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TABLE I.
Oxygen Uptake of Chopped Rat Brain in Various Media Expressed in μl/mg/hr (Wet Wt).

Additions to medium	Observations	Mean ± stand. dev.	Significance of difference from 1) Control	2) Bilirubin
1) None—control	22	.663 ± .065		
2) Bilirubin	31	.490 ± .066	$p < .001$	
3) Biliverdin	9	.623 ± .035	$p > .05$ (not sig.)	$p < .01$
4) Bilirubin + cytochrome C	8	.689 ± .094	$p = .5$ (not sig.)	
5) Cytochrome C	8	.733 ± .048	$p < .05$	$p < .01$
6) Bilirubin + methylene blue	6	.680 ± .106		$p < .01$

experimental solutions were adjusted with HCl to a pH similar to each other within 0.05. Osmolarity, estimated by determining the freezing point, was adjusted with distilled water to within 4 milliosmoles. From day to day, larger variations were permitted, pH varying from 7.9 to 8.3 and milliosmolarity from 265 to 291. Variations of this size did not affect the results appreciably. The substances tested consisted of bilirubin, oxidized bilirubin ("biliverdin"), cytochrome C and methylene blue. The bilirubin was obtained from the Amend Drug and Chemical Co., N. Y. City, lot No. C79142. This bilirubin is indirect. The cytochrome C was obtained from the Treemond Co., N. Y. City, lot No. 3719. Oxidized bilirubin, presumed to be biliverdin because of its green color, was prepared by bubbling oxygen through the bilirubin solution in a bath of boiling water for 3 hours. Neither oxidation with oxygen, nor the addition of cytochrome C in a 10^{-4} M concentration resulted in any visible precipitation. Methylene blue, however, when mixed with bilirubin causes a fine granular precipitation.

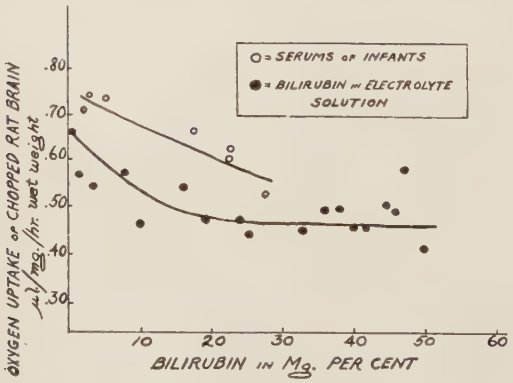

FIG. 1.

Results. The results appear in the Table and in the Figure. The Figure suggests that above about 25 mg % of bilirubin, the degree of respiratory inhibition becomes constant. Accordingly, for statistical validation of the depression in Qo_2 the comparison is made between the oxygen uptake by brain in the control solution and that in bilirubin solutions varying in concentration from 20 to 55 mg %. Twenty-two control flasks yielded a Qo_2 of 0.663 ± 0.065 (mean ± S.D. of the series) μl per mg per hour. Thirty-one flasks containing bilirubin solutions yielded a mean value of 0.490 ± 0.066 μl/mg/hr. The difference between these 2 means is 9.6 times its standard error, so that p is less than 0.01. The difference is highly significant.

Nine experiments using "biliverdin" yielded a mean of 0.623 ± 0.035 μl/mg/hr, a value slightly less than that for the controls, but not quite significantly so. Whether the slight apparent depression of Qo_2 by biliverdin is the result of experimental error, of contamination of the sample with bilirubin or depends upon a slight action by biliverdin itself is not apparent from these experiments. The difference in action between bilirubin and biliverdin, however, is highly significant, being 8 times its standard error ($p < 0.01$).

Cytochrome C added to the bilirubin solution in a concentration of 10^{-4} M increased oxygen consumption to a level slightly above that of the controls. Cytochrome C, when brain tissue is present, converts bilirubin to biliverdin. This oxidation presumably involves cytochrome oxidase from broken cells,

since cytochrome C alone does not accelerate the conversion of bilirubin to biliverdin when the solution stands at room temperature. However, even when brain tissue is present, the conversion to biliverdin is slow, a moderate amount of green color being present after 2 hours. In contrast with this slow action, the effect of cytochrome C on the bilirubin inhibition of oxygen uptake is noticed in the first period after the introduction of the cytochrome. Inspection of the flasks showed no visible green at this time, and even after 1 hour there was very little green to be seen. Cytochrome C alone raised the respiration of brain to 10% above the control rate, whereas the bilirubin solutions to which cytochrome C had been added supported respiration of the brain at a rate 43% greater than when the bilirubin was not accompanied by cytochrome C. A possible interpretation is that cytochrome C overcomes the inhibition by bilirubin of part of the oxidative process.

Methylene blue in equimolar concentration with that of the bilirubin reverses the inhibition, but the action is not immediate. In fact, the methylene blue must be mixed several hours prior to an experiment. During this period, there is a slow conversion of bilirubin to biliverdin, and, in addition, a precipitate forms consisting of fine blue-green granules. When added to the control flasks, methylene blue induced no change in Q_{O_2} in 4 observations.

In addition to the above, observations have been made with rat brain bathed in serums from newborn infants with varying degrees of indirect bilirubinemia. The results are indicated by the open circles in the Figure. Each point is the average of from 2 to 4 flasks. There were 9 flasks with serum bilirubin concentrations of 7 mg % or less and 9 with concentrations of from 17 to 27 mg %. Comparison of these two sets of flasks discloses that the probability of the difference between the two being the result of chance is 0.05. Further work with heavily jaundiced serums from infants is needed, but because of the use of replacement transfusion, such serums are scarce in this clinic.

Three observations were made with commercial bilirubin purified by crystallization, and 2 observations with bilirubin purified by extraction with chloroform and washing with water. These preparations gave results identical to those with the product as obtained from the supply house.

Two observations using liver slices showed a depression of Q_{O_2} by bilirubin similar to that found with brain. It is presumed that the liver slices could be cut with less trauma to the cells than might be expected by the process of chopping the brain. Additional evidence that bilirubin can exert its action on intact cells is obtained from the fact that no greater inhibition was obtained in two observations in which a brain homogenate was used. A final answer to the question of whether intact cells can be affected by bilirubin, however, awaits *in vivo* observations.

Comment. These observations indicate that bilirubin depresses the respiration of brain *in vitro*. It seems likely, though not certain, that this action can be exerted upon intact cells. It is of interest to note that Najjar(8) has postulated that bilirubin might have a toxic action upon intact cells.

It remains for future work to show whether the phenomena described in this report also take place in the living animal. One does not know what the margin of oxidative safety for a neuron is. One might postulate the participation in the infant of other factors leading to tissue anoxia, such as anemia, incomplete expansion of lungs, sludging of red blood cells, and depression of enzyme systems by excessive use of therapeutic oxygen. Were the respiratory center depressed by the action of these factors combined with the inhibitory effect of bilirubin, a vicious cycle would be set up. In support of such a theory is the observation in this clinic that respiratory rates are very slow for many hours prior to death from kernicterus.

The confinement of kernicterus to the newborn period is another fact which is not explained by the theory that bilirubin is the toxic agent. The blood-brain barrier is known to be less resistant to trypan blue(9) and to bilirubin(10,11) in young animals than in older ones. The rapid maturation of cellular enzyme systems shown by Potter and coworkers(12) might also have some bearing

on the age incidence of kernicterus. These subjects need more attention than they have been so far accorded.

Summary and conclusions. Bilirubin added to the ambient fluid in concentrations above 20 to 25 mg % depresses the respiration of chopped rat brain by approximately 25%. This inhibition can be counteracted by oxidation of the bilirubin and by the action of methylene blue on the bilirubin. Cytochrome C also reverses the inhibition. The mechanisms of these reversals appear to be different.

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Urinary Estrogens and Related Compounds in Postmenopausal Women With Mammary Cancer: Effect of Cortisone Treatment. (20850)

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This paper presents data on the quantification of estrogens and related compounds in the urines of 15 postmenopausal women. Eight were normal and 7 had cancer of the breast. The effect of cortisone therapy upon urinary estrogens was investigated at various times in 4 of the 7 cancer patients. Observations were also made concerning the influence of advancing disease and of the surgical removal of X-rayed ovaries.

Methods. From the 8 normal women 72- to 96-hour specimens were analyzed while only 24- to 48-hour collections were available on the cancer patients. The accuracy of each collection was checked by creatinine determination and the volume corrected when necessary. One aliquot of each specimen was subjected to the usual HCl hydrolysis(1) and a second to Zn-HCl hydrolysis(2) prior to continuous benzene extraction(1) for bioassay. The total estrogenic activity released by these 2 procedures is designated T_o and T_{zn} respectively. The residual urines after benzene extraction of the HCl hydrolyzed aliquots were further studied by methods previously described(3) in order to determine how much of the difference between T_o and T_{zn} values might be accountable to more complete hydrolysis of conjugated estrogens. The results of these latter studies are not included in this report since in no instance could the difference observed between T_o and T_{zn} values be explained on this basis. It would appear to be safe to assume that the very high T_{zn} values found in many of the cancer urines represent unknown constituents, possibly estrogen oxidation products or precursors(3), related to the estrogens but exhibiting estrogenic activity only after Zn-HCl treatment. T_o values, on the other hand, represent estrogens excreted as such.

Bio-assay was performed by a standardized procedure(3) on spayed mature female rats. Unless more than 50 I.U. of T_o or more than

TABLE I. Estrogens in Urine of Women without Ovarian Function. Effect of mammary cancer and cortisone therapy.

Group	No. of cases	No. of urines	Urinary estrogens (I.U./24°) — HCl hyd. Range	HCl hyd. Avg*	Zn-HCl hyd. Range	Zn-HCl hyd. Avg*
Normal, postmenopausal	4	10	25- 50	37	50- 100	75
" , ovariectomized	4	10	12- 50	30	25- 100	75
Cancer, X-ray castrated						
Not receiving cortisone	5	6	<50-385	138	<100-1960	640
During cortisone therapy	4	6	<50- 80	13	<100- 575	158
Cancer, ovariectomized						
Not receiving cortisone	4	5	<50-125	25	<100-4700	1370
During cortisone therapy	2	3	<50	<50	<100	<100

* Because of the wide range of observed values in urines of cancer patients, the average figures presented in this table merely show the trends of excretion rates in these 4 groups. Individual value for each "cancer" urine is given in Table II. When the activity of a specimen was established at less than 50 I.U./24 hr volume, that value was considered as zero when calculating the avg. This, of course, lowers the avg figures, but does not detract from the significance of the trends demonstrated in this table, especially when one considers the range of values in each group and the very large differences observed between the groups being compared.

100 I.U. of T_{zn} are present per 24-hour volume of urine, the end-point criteria of this method cannot be met in analyses of less than 72-hour collections. Negative values at these levels can be established, however, and are so expressed in Tables I and II.

Results. In the urines of normal women whose ovaries have been removed or who have passed the menopause we have never found more than 50 I.U. of estrogenic activity per 24-hour volume after HCl hydrolysis (T_o) or more than 100 I.U. after Zn-HCl hydrolysis (T_{zn}). This was true of the 20 specimens collected from the 8 control women of this study. The findings in these and in 20 specimens from the 7 women with mammary cancer are summarized in Table I. When not receiving cortisone, the average output of estrogens excreted as such (T_o) by cancer patients with X-rayed ovaries was about 4 times as high as the average output by normal postmenopausal women. This was not true in the cancer patients whose ovaries had been surgically removed. Only one of these had an abnormally high level of T_o. The pretreatment output of the unknown compounds (T_{zn}) that may represent estrogen oxidation products or precursors, however, averaged 9 times the normal in the cancer patients with X-rayed ovaries and 18 times the normal in the cancer patients who had been oophorectomized. In both types of cancer patients these very high levels of excretion, both of the estrogens excreted as such and of related compounds, were markedly lowered by cortisone therapy. They were brought to within the normal range or below in all but 2 of 9 experiments (Table II).

In Table II the findings on the 7 cancer patients are presented in sufficient detail to demonstrate in the individual cases not only the effect of cortisone therapy but also the influence of advancing disease and of the surgical removal of X-rayed ovaries. Higher excretion rates with advancing disease were apparent in studies on all three of the patients who were followed over long periods of time. Mrs. E., whose output of T_o and T_{zn} were well above normal 2 years after X-radiation, was found to have an even higher level of excretion in both fractions 17 months later when her disease had become more advanced. In Mrs. C. and Mrs. B. the effect of surgical removal of X-rayed ovaries as well as of advancing disease was observed. After oophorectomy the high levels of estrogens excreted as such (T_o) disappeared from the urines of both of these women, while the T_{zn} values on both rose with advancing disease despite the absence of any ovarian tissue. This was especially striking in the case of Mrs. C. By April, 1953, 7 months after oophorectomy, she had very widespread cancer with pain. Her urinary output

TABLE II. Estrogens in Urine of Postmenopausal Women with Mammary Cancer.

		Not receiving cortisone			During or just after cortisone treatment				
			Urinary estrogen (I.U./24°)			Cortisone given		Urinary estrogen (I.U./24°)	
Patient	Condition of ovaries	Date of urine	T_o	T_{zn}	Date of urine	Daily dose, mg	Duration of dose	T_o	T_{zn}
#1 Mrs. E., aged 43-46	X-ray cast. in 1947	3/20/49	100	425	8/18/51	25	7 days	<50	<100
		8/10/51	165	665	10/18/51	25	2 mo	<50	<100
						200 to			
#2 Mrs. C., aged 42	X-ray cast. in 1948	7/ 2/52	125	665	7/10/52	50	7 days	<50	375
					9/11/52	25	2 mo	<50	<100
#3 Mrs. B., aged 37-38	X-ray cast., Oct. 1951	12/ 9/51	60	125	10/ 6/52	25	10 "	<50	<100
#4 Mrs. V., aged 50	X-ray cast., 1950	9/29/53	385	1960	11/16/53	75	5 wk	80	575
#5 Mrs. L., aged 48	X-ray cast., May 1952	10/30/52	<50	<100					
						150 to			
#2 Mrs. C. (cont.)	Oophorect., Sept. 1952*	10/18/52	<50	750	10/ 5/52	25	3 mo	<50	<100
		4/24/53	<50	4700	5/14/53	75	3 wk	<50	<100
#3 Mrs. B. (cont.)	Oophorect., April 1953*	6/22/53	<50	250	7/ 5/53	75	2 "	<50	<100
#6 Mrs. M., aged 52	Oophorect., Feb. 1953*	4/28/53	125	1150					
#7 Mrs. S., aged 33	Oophorect., Feb. 1953	5/ 7/53	<50	<100					

* Sections of these ovaries were examined by Dr. George V. Smith and found to exhibit typical stromal hyperplasia as first described by him(10) in the ovaries of postmenopausal women with cancer of the endometrium.

of estrogens excreted as such was not elevated as it had been on July 2nd, 1952, before oophorectomy. Her urinary T_{zn}, on the other hand, was 7 times as high as it had been then and 60 times as high as the average level in normal surgical castrates. Cortisone therapy was particularly effective in relieving the pain in this patient. After 3 weeks on cortisone her T_{zn} value was within or below the normal range. In only one patient of this study did cortisone therapy for 2 weeks or more fail to eliminate excessive excretion of estrogens and related compounds. Mrs. V., an X-ray castrate, had received the same dosage of cortisone as Mrs. C. and for an even longer period of time by 11/16/53. Her urine still contained excessive amounts of estrogens excreted as such and of urinary products rendered estrogenic by Zn-HCl treatment, although the levels of both were considerably lower than they had been before treatment. It is perhaps worthy of note that this patient was the only one of the 4 treated cases who derived no definite relief of pain from cortisone, as well as the only one whose urinary output of T_o and T_{zn} was not lowered to within or below normal limits by this form of therapy.

Discussion. That postmenopausal women with cancer frequently excrete more estrogen than normal postmenopausal women was reported by Pincus and Graubard(4) and has recently been confirmed by Dao(5) in patients with widespread mammary cancer. In 12 ovariectomized women who had been adrenalectomized for the treatment of breast cancer, however, Dao found no urinary estrogen. Since these patients were being maintained on cortisone, this treatment rather than adrenalectomy might have accounted for the absence of demonstrable estrogen in their postoperative urines. The adrenals, however, are logically assumed to be the source of urinary estrogen in males and in women without ovarian function. High titres have been observed in ovariectomized women following major surgery(6) and in the urines of males after severe burns(7), the inference being

that any condition of stress, with resultant hyperactivity of the adrenal cortex, may be accompanied by increased secretion of adrenal estrogen. The finding herein reported that cortisone suppresses estrogen excretion further implicates the adrenals since cortisone inhibits secretory activity in the human adrenal cortex (8). The findings herein presented also suggest, however, that X-rayed ovaries as well as the adrenals may be a source of estrogen in women with mammary cancer. Here again a condition of stress may be involved since pituitary release of gonadotropic as well as adrenotropic hormones has been demonstrated in animals in response to noxious stimuli(9). The stromal hyperplasia observed by Dr. George V. Smith in the ovaries of these patients (Table II) is of particular interest in this connection, especially since it is known to occur very much more frequently in the postmenopausal ovaries of women with cancer than in those of normal postmenopausal women(10,11). It has yet to be demonstrated, however, that stromal hyperplasia represents estrogen secretion. Another possibility is that it may influence the metabolism of adrenal estrogen or related compounds so that more is excreted in estrogenically active forms. It is tempting to postulate that high T_{zn} titres in these patients reflect adrenal secretion of estrogen precursors, but in our present state of knowledge any interpretation of these data must be purely speculative. The rising titres with advancing disease are in keeping with the hypothesis, however, that, whatever the source of the urinary estrogens and related compounds, metastatic cancer is in some way responsible for the excessive amounts observed.

Summary. In 7 postmenopausal women with mammary cancer the urinary output of estrogens and related compounds has been studied under various conditions and compared with that of 8 normal postmenopausal women. Five of the 7 cancer patients were found to excrete excessive amounts. In these 5, when not receiving cortisone, the output of estrogens excreted as such averaged nearly 5 times the mean normal, and that of compounds estrogenic after Zn-HCl hydrolysis averaged 16 times the mean normal. Cortisone therapy markedly lowered these titres and reduced them to normal levels in 3 of 4 patients so treated. Except when under cortisone treatment the titres of estrogen and related compounds rose with advancing disease. The surgical removal of X-rayed ovaries lowered the titres of estrogens excreted as such but not of the unknown compounds rendered estrogenic by Zn-HCl treatment.

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Summation and Tetanus in Cardiac Muscle. Effects of Temperature, Epinephrine and Digitoxin.* (20851)

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It is generally recognized that fundamental understanding of the mechanisms of cardiac weakness and failure must rest upon knowledge of the contractile properties of cardiac muscle. Information regarding differences and similarities between the characteristics of cardiac and skeletal muscle is of importance in this regard. While it is usually stated that cardiac muscle does not exhibit the phenomena of summation and tetanus, reports in both the older(1-3) and more recent(4,5) literature indicate that such responses are at least potentially possible. It is the purpose of this report to confirm the occurrence of summation and incomplete tetanus in the isolated cardiac tissue of the frog and rat under stated conditions and to present data on the mechanism of its production and its modification by certain cardioactive substances in the case of the frog.

Methods. Ventricular strips were prepared from the mid-portion of the ventricle of *Rana pipiens,* and suspended in oxygenated Ringer's solution. The base of the muscle was held in a specially designed clamp into which were incorporated a pair of chlorided silver electrodes. The upper end of the strip was connected to a Statham strain gauge (Model 67-0.3-800) whose output was amplified and recorded on a Grass ink-writing oscillograph. Contractions were essentially isometric and were recorded with the muscle under an initial tension of 500 mg. Stimuli were applied from a square wave stimulator either directly to the base of the muscle through both clamp electrodes or with one of the clamp electrodes serving as cathode and the bath serving as anode. Prior to use the animals were kept in running water at temperatures of 18-22°C. The experiments were performed during the period from February to June. Right ventricular strips were prepared from albino rats of the Holtzman-Rolfsmeyer strain after the method of Feigen *et al.*(6). Animals were killed by a blow on the head and the strips suspended in buffered medium(6) within 1-2 minutes of the time of death. The bath was gassed with 95% O_2-5%CO_2 and maintained at a temperature of 38°C. Suspension, stimulation and recording were the same as already described for the frog. In both frog and rat experiments strips were driven at a basic rate of one beat per second and were allowed to equilibrate for an initial period of one-half hour before experimental observations were begun. Details of experimental procedures will be presented with results.

Results. The classical response of frog cardiac muscle to repetitive stimulation is apparent in Part A of Fig. 1. At a temperature of 22°C an increase in the rate of stimulation from one pulse per second to 5 pulses per second resulted in a decrease in the magnitude of the individual twitches, and a reduction in the total tension developed by the strip during the period of rapid stimulation. More rapid rates of stimulation produced similar responses. There is no evidence of summation of individual contractions. If the temperature of the bath is raised to 30°C, however, the response pictured in Part B of the record is obtained. Stimulation at a rate of 5 pulses per second now results in a reduction in the magnitude of the individual contractions, but the diastolic baseline rises and

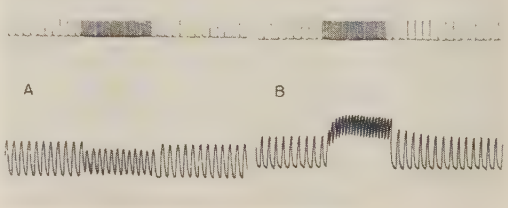

FIG. 1. Production of incomplete tetanus in frog ventricle strip by elevation of temperature. Stimulus record above, myogram below.
A—temp. 22°C B—temp. 30°C

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the total tension developed by the strip during the period of rapid stimulation exceeds that produced at the slower rate of stimulation. Summation of contractions is apparent and the record is comparable to that of incomplete tetanus in skeletal muscle. Upon cessation of rapid stimulation the baseline returns to its previous level and the magnitude of the individual beats likewise returns to pre-tetanic levels. It is of interest to note that the first few beats following cessation of rapid stimulation are, however, of increased amplitude. Experiments on 30 preparations indicated that the phenomenon could be consistently reproduced, the temperature necessary for its production varying from 26°C to 34°C with an average of about 30°C. Frequencies of 5 to 10 per second were optimal for its production. These results clearly indicate that summation and incomplete tetanus in frog cardiac muscle strips can be regularly produced by elevation of bath temperature.

Since the phenomena of summation and tetanus would seem to be related to the well known occurrence of treppe in cardiac muscle it was of interest to study the effect of various cardioactive substances on the phenomenon in the light of the recent reports of Hajdu and Szent-Györgi(7,8). Substitution of serum for Ringer's solution in the bath was without effect, and reduction in K ion concentration produced inconsistent results.

Addition of epinephrine hydrochloride (Parke-Davis) to the bath in concentration of 10^{-6}, consistently abolished the tendency for cardiac strips to summate at high temperatures. The records of such an experiment are reproduced in Fig. 2. Part A shows the incomplete tetanus produced by stimulation at the rate of 5 per second at 32°C and the almost completely fused response to 10 stimuli per second at this temperature. Addition of epinephrine to the bath one minute prior to the record of Part B resulted in abolition of the summated response. There is a slight elevation of diastolic baseline but there is no summation of beats during the period of rapid stimulation and the tension developed during this period is less than that developed at a driving rate of one per second. Part C indicates the results of tetanic stimulation 5

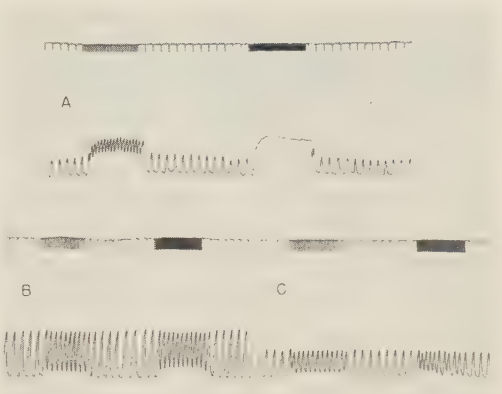

FIG. 2. Inhibition of summated response by epinephrine. Temp. 32°C. Stimulus record above, myogram below.
Part A—Stimulation at 5 and 10/sec.
B—One min. after addition of 10^{-6} epinephrine to bath.
C—Five min. after addition of epinephrine.

minutes after the administration of epinephrine. Although the positive inotropic action of the drug has markedly diminished, there is no summated response to stimuli at 5 or 10 per second. Following washing out of the drug with Ringer's solution the summated response could be once more obtained (not shown in this figure).

Hajdu and Szent-Györgi(8) reported that digitalis glycosides abolished the treppe response in frog heart and suggested that action of these substances on the permeability of the muscle membrane to K ions was responsible for their effects. We accordingly investigated the influence of digitoxin (Digitoxin, Abbott) in concentration of 0.3 μg per ml on the development of summation and tetanus in frog ventricle strips. Observations at temperatures of 30°C and above suggested that the glycoside did not abolish, but actually favored the development of the summated response. This effect was clearly demonstrated by the development of summation and tetanus in digitalized strips at temperatures at which untreated strips failed to show these responses. The records of such an experiment conducted at 22°C are reproduced in Fig. 3. Within 5 minutes of the administration of the drug the unsummated response to repetitive stimulation at 5 beats per second was converted to an incomplete tetanus, and after 15 minutes the

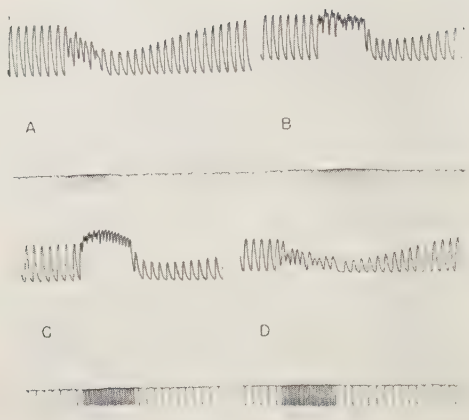

FIG. 3. Production of summated response by digitoxin. Temp. 22°C. Myogram above, stimulus record below.
A—Untreated strip.
B— 5 min. after digitoxin, 0.3 μg/ml.
C—15 min. after digitoxin.
D—30 min. after washing with Ringer's.

response was well established. Washing with Ringers gradually removed the effects of the drug and the muscle responded in the untreated fashion. Re-digitalization resulted in a return of the summated response.

It was of interest to determine whether the inhibitory action of epinephrine on summation and tetanus at higher temperatures also extended to that produced as a result of digitalization. Addition of epinephrine to digitalized strips consistently abolished the summated response.

In an approach to an understanding of the observations described, modification of the duration of the refractory period of the muscle strips offered a possible explanation. Measurements were accordingly made, the refractory period as here defined being the minimal time interval between the driving stimulus at a rate of one pulse per second and a supramaximal test shock of 14 milliamps magnitude, necessary to produce a detectable mechanical response. In all cases, development of summated responses at elevated temperatures was associated with shortening of the refractory period and abolition of these responses by cooling was accompanied by lengthening of refractoriness. Typical changes in refractory period induced by digitoxin and epinephrine are graphically summarized in Fig. 4. It can be seen that the development of summation as the result of digitoxin action is associated with reduced refractoriness and the inhibitory action of epinephrine on this phenomenon with increased duration of the refractory period. These findings are in agreement with classical teaching that the failure of cardiac muscle to summate under usual conditions is a function of the length of its refractory period, and with reports in the older literature(1,2) in which cardiac tetanus was produced by procedures which shortened the refractory period. The reduction in duration of the refractory period noted with digitoxin is in agreement with the observations of Macleod(9) and with the recent report of Woodbury and Hecht(10) on the effects of cardiac glycosides on single ventricular fibers. The contrary action of epinephrine is compatible with reports indicating slowing of repolarization in cardiac muscle under the influence of this agent(11,12), and the associated inhibition of the summated response confirms the findings of Spadolini(13) in the case of toad ventricle. It is of interest that these 2 potent cardio-active substances, each capable of increasing the strength of cardiac contraction, should show antagonistic properties in relation to the duration of its

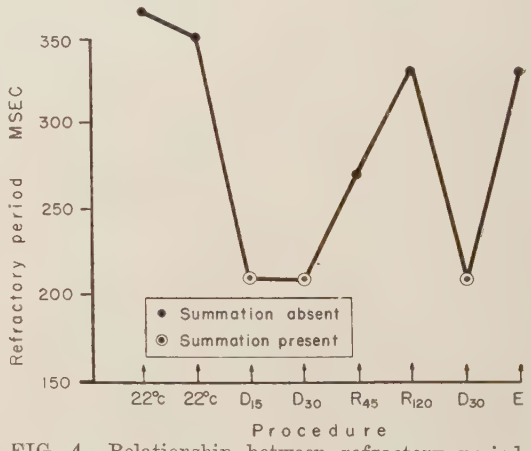

FIG. 4. Relationship between refractory period, summation and action of digitoxin and epinephrine. Temp. 22°C.

D 15 =	15	min. after	digitoxin
D 30	30	" "	"
R 45	45	" "	wash out with Ringers
R 120	120	" "	" " " "
D 30	30	" "	redigitalization
E	1	" "	epinephrine

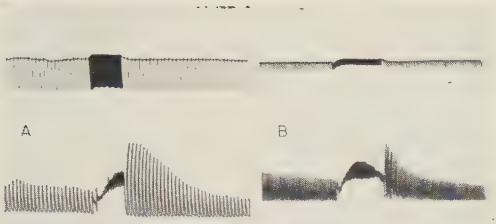

FIG. 5. Summation and post-tetanic potentiation in rat ventricle strip. Stimulus record above, myogram below. Response to stimuli @ 40/sec.
A—After basic rate of 1/sec.
B— " " " " 5/sec.

refractory phase.

While frog ventricle strips could be shown to demonstrate summated responses only under the conditions stated above, rat ventricular strips demonstrated the phenomenon at normal temperature (38°C) and in the absence of treatment with drugs. A typical response is pictured in Fig. 5. Increase in the rate of stimulation from one beat per second to 40 beats per second resulted in summation and incomplete tetanus in this case and in marked post-tetanic potentiation of contractions. Since the normal heart rate of the rat is in the neighborhood of 300 to 400 beats per minute, the experiment was repeated with a driving stimulus of 5 per second. Again, increase in rate of stimulation to 40 per second produced the summated response and post-tetanic potentiation. In contrast with the frog, frequencies of stimulation necessary to produce summation are higher in the rat, 20-40 stimuli per second being optimal, and the response is somewhat less consistent. The results, however, confirm the reports of Robb(5) and of Di Palma and Mascatello(4) on the occurrence of summation in mammalian cardiac muscle.

In view of the observations on the frog it is tempting to consider change in duration of the refractory period as the primary factor in the occurrence of summation and tetanus in rat cardiac muscle. Preliminary experiments indicate that this may not be wholly the case. Of 12 preparations tested for the summated response, 8 consistently gave positive results. No correlation existed between the duration of refractory period and the presence or absence of summation. Moreover, while administration of epinephrine abolished the summated response in all but 3 of these strips, this effect was accompanied by only small and inconsistent changes in refractory period. While these observations are as yet incomplete, they suggest that the operation of other mechanisms, such as the recruitment of contractile elements suggested by Robb(5) may play an essential role in the summation of contractions in rat cardiac muscle with its normally short refractory period.

Summary. 1. Summation of contractions and incomplete tetanus may be consistently produced in frog ventricle strips by elevation of bath temperature. In these experiments a temperature of about 30°C regularly induced the summated response. 2. Digitoxin in conc. of 0.3 μg/ml potentiated the summated response and caused its appearance at lower temperatures. 3. Epinephrine in conc. of 10^{-6} prevented summated response to digitoxin or high temperature. 4. The summated response to digitoxin or high temp. is associated with reduction in duration of refractory period and the inhibitory effect of cooling or epinephrine with increase in duration of refractoriness. 5. Rat ventricle strips may show summation, incomplete tetanus and post-tetanic potentiation at temp. of 38°C and in the absence of treatment with drugs. The mechanisms of these phenomena are not yet delineated.

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Isolation of an Agent Causing Bilirubinemia and Jaundice in Raccoons. (20852)

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An agent causing bilirubinemia, jaundice, and frequently death in raccoons (*Procyon lotor*) has been isolated from the liver of a sick raccoon trapped in Maryland. This agent is considered to be a virus as tests for bacteria and for leptospirae have been negative and it is filterable. Thusfar, there has been no indication of relationship between this agent, which will be designated Raccoon Jaundice Virus (RJV) and other viruses, including those of feline distemper, canine distemper and canine hepatitis. RJV may be responsible for epizootics which occasionally affect raccoon populations. The present report describes a few attributes of the agent and of the disease which it produces in raccoons as observed in 5 continuous passages.

Materials and methods. Raccoons used for experiments were live-trapped in Maryland or obtained from a dealer in Florida. They were maintained on a diet of horse meat plus bread, lettuce, apples, and bananas. Preparations of the agent were from livers of diseased raccoons, made into 10% suspensions with meat infusion broth. Such suspensions were stored in a dry ice cabinet in sealed glass ampoules. Unground portions of infected livers were also stored at –40°C in an electric deep freeze. Both methods of storage were effective. In routine passages, raccoons were given 1 ml of a 10% suspension intraperitoneally.

Description of disease. Two isolations of the agent (RJV) were made on first passage from the original raccoon, one from the liver and one from the kidney. This raccoon was weak and had exudate about the eyes when first observed. On necropsy an empty intestinal tract but no jaundice or gross pathology of any kind was encountered. This original animal had convulsive movements of head and paws, signs which have not been observed in inoculated animals. Raccoons inoculated intraperitoneally with suspensions of infected liver usually became ill within 9 to 14 days. In 5 continuous passages, virulence remained high and almost all raccoons not killed when severely ill died from the disease. Illness began with cessation of feeding and was often accompanied by appearance of exudate about the eyes; sick animals remained quiet, eating little or nothing. A few had a bad smelling, scanty anal discharge. Vomiting was not observed. After 5 days, jaundice was sometimes detectable in the gums. Almost all inoculated raccoons, if not sacrificed earlier, were dead or moribund within 4 to 8 days of the time illness was first observed. On necropsy raccoons ill 5 or more days usually had ample stores of fat, although intestinal tracts were empty. Judged by full urinary bladders often encountered and the fact that sick raccoons drank freely, the animals were not dehydrated. Jaundice frequently was of an intense degree in raccoons severely ill or recently dead from the disease. In other animals, jaundice was slight or absent in spite of an elevated serum bilirubin (Table I) and a positive foam test in the urine. Neither petechiae nor hemorrhages were observed. Liver, spleen, kidneys, intestinal tracts and other organs appeared normal, except that occasionally sick animals had small patches of pulmonary consolidation. Histologic studies, to be reported later when more material has been accumulated, have so far given scanty evidence of pathologic changes in liver, kidneys, or other organs. Pathologic changes were found in the liver of one raccoon which had a total serum bilirubin of 11 mg %, the highest level encountered in any animal. These changes consisted of necroses of single liver cells widely scattered throughout the parenchyma. There was no attendant inflammatory reaction. Some few raccoons developed purulent con-

TABLE I. Quantitative Serum Bilirubin Determinations in Relation to Course of Hepatitis* in Raccoons and a Ferret Inoculated Intraperitoneally.

Animal No.	Day of disease	Quantitative serum bilirubin 1 min.	Total	Clinical icterus	Clinical condition	Incubation, days	Inoculum
732	†	.04	.16			21	Kidney unfiltered, 1st passage
	5	.18	1.06				
	8	1.59	3.34	Present	Moribund, killed		
842	†	.01	.17			8	Liver unfiltered, 2nd passage
	3	.16	.11				
	7	1.44	2.38	None	Died under observation		
846	†	.12	.11			14	Liver filtered, 3rd passage
	6	2.95	4.56	"	*idem*		
803	†	Too fatty	.05			12	Liver filtered, pool of passages 1, 2, and 3
	7	2.35	5.38	Present	Moribund, killed		
717‡	†	.12	.13			12	Liver filtered, 3rd passage
	2 (?)	1.48	2.20	None	*idem*		

* Tests in laboratory of Dr. Roderick Murray. Biologics Control, Laboratory of Infectious Diseases, National Microbiological Institute.

† Pre-inoculation.

‡ Ferret.

junctivitis with gluing of the eyelids. This complication was presumably due to secondary invasion by pyogenic organisms, for it even appeared in one raccoon fully protected from bilirubinemia by immune serum.

Laboratory findings. As shown in Table I in the case of a few animals used as examples, elevation of serum bilirubin has been marked in diseased raccoons and in a single ferret. Hematocrits run on diseased or dying raccoons have averaged between 42 to 45% and, in absence of signs of dehydration, support the conclusion that anemia, hemolytic or otherwise, plays no role in the disease picture, especially as blood smears have appeared normal. Leucocyte counts made for 5 sick raccoons were not remarkable. Raccoon 846 (Table I), for example, had a total white cell count of 16,400 cu mm, of which 72% were neutrophiles, 13% lymphocytes, 10% monocytes, 1% eosinophiles, and 4% young forms, when moribund on its 6th day of disease.

Transmission. The raccoon disease caused by (RJV) has been passed parenterally by suspensions of infected liver, kidney, spleen and on one occasion by blood obtained on the first day of illness and passed directly to a recipient raccoon by intraperitoneal inoculation. This last animal became ill in 13 days. Although most passages were made by the intraperitoneal route, one raccoon became ill 11 days after subcutaneous inoculation. In other experiments, confined to single animals, oral transmissions were not successful with eye exudate and saliva, stool specimen from a raccoon on the 2nd day of disease or from a dilute suspension of infected liver given by stomach tube. At the outset of present investigations one raccoon appeared to have developed bilirubinemia from spontaneous transmission. As no other cases of this sort have arisen among inoculated raccoons or among uninoculated animals kept for many weeks in the same room as controls, it is felt that precautions taken have been effective. These precautions have included placing actually diseased raccoons on bottom shelves in one room and spacing cages of other experimental animals well apart on upper shelves. In special experiments raccoons have been maintained in separate laboratory rooms.

Neutralization tests. In these tests 4 sera were heated at 56°C for ½ hour, then mixed with an equal volume of 10% liver suspension which had been centrifuged at 4000 rpm for 1 hour. These mixtures were inoculated separately into peritoneal cavities of 7 young raccoons in amounts of 1.5 ml. Two of these raccoons which had received pre-inoculation phase serum from raccoon No. 842, became severely ill and had elevations of serum bili-

TABLE II. Results of Neutralization Tests Performed with Raccoon Sera in Raccoons Inoculated Intraperitoneally with Serum-Virus Mixtures.

Raccoon	Serum phase	No. of raccoons tested	Fate of test raccoons
842	Pre-inoculation	2	Severe illness + bilirubinemia
842	7 days after onset of illness	2	Remained well
846	*idem*	1	*idem*
829	Convalescent	2	"

rubin when killed (Table II). The other raccoons never became ill and were apparently fully protected by sera from 2 raccoons (No. 846 and No. 842) which had had illnesses of 7 days duration, as evidenced by jaundice at necropsy. Convalescent serum from a third raccoon (No. 829) was also protective on 2 occasions. In additional tests with commercial sera one raccoon was not protected by a combined anticanine distemper, anti-canine hepatitis serum, and 2 raccoons were not protected from death by anti-feline distemper serum.

Properties of agent. Preparations of the agent have survived storage in a dry ice cabinet for 6 months and liver suspensions frozen and thawed twice, and in one instance, 4 times, have remained infective. Three successive passages were carried out with liver suspensions passed through Selas .03 filters shown to be bacteria tight. As leptospirae can cause hepatitis and also pass through such filters, special efforts were made to determine whether they might be involved. Convalescent serum from raccoons No. 829, which had neutralizing antibodies as shown in Table II, were negative in complement-fixation and in agglutination tests for leptospirae.* Leptospirae could not be cultured from infected raccoon livers in Shüffner's modification of Vervoort's medium. Furthermore, leptospirae could not be found when histologic sections from diseased raccoon livers were stained with a spirochaetal strain shown to be effective in control tissues known to contain leptospirae. It is concluded, therefore, that this agent is a virus.

Host range. Ferrets are susceptible to infection with (RJV) as shown in Table I for Ferret No. 717. This animal, when found moribund 12 days after inoculation, had experienced a rise of serum bilirubin in the course of its disease. When passed to raccoons, suspensions of this ferret's liver resulted in bilirubinemia and jaundice. Animals inoculated without evidence of a resultant disease included suckling and weanling mice and hamsters, rats, cotton rats, rice rats, deer mice (Peromyscus) domestic rabbits, cottontail rabbits, guinea pigs, 4 puppy dogs, 5 Rhesus monkeys, 2 suckling pigs and embryonated eggs.

Discussion. The present report demonstrates that raccoons are susceptible to a disease which, under laboratory conditions, is highly fatal and is accompanied by bilirubinemia, jaundice, and conjunctivitis. As the etiologic agent is filterable and resistant to repeated freezing and thawing, it appears to us to be a virus, especially as tests for leptospirae have been negative. Whether it is a hitherto undescribed virus or is related to one already known in some other host cannot be stated at the present time.

In the disease of raccoons described above, jaundice has appeared to be due primarily to liver dysfunction rather than to any hemolytic type of anemia. A puzzling feature of the disease, however, has been the meagerness of pathologic findings considering the severity of the disease. It is hoped that continued study of the disease under varied conditions including intracerebral inoculations will make possible a more complete description of histologic changes which must take place in such a severe and fatal disease. Of other mammals so far studied, only the ferret has developed bilirubinemia and jaundice similar to that found in raccoons. Efforts are also being made to find out what relationship, if any,

* Tests made at Army Medical Service Graduate School, Veterinary Division, Washington, D. C.

raccoon hepatitis, if such it is, may bear to infectious hepatitis of man.

Summary. An infectious agent, which appears to be a virus (RJV) has been isolated from the liver of a wild raccoon which has led to a highly fatal type of disease characterized by conjunctivitis and an elevated serum bilirubin frequently accompanied by jaundice on inoculation of raccoons. Ferrets also appear to be susceptible to infections with this agent.

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Calcification. XII. Cation-Linked Inhibition by Fluoride and Cyanide Ions in β-Glycerophosphate Medium.* (20853)

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In a previous report it was shown that in the presence of magnesium ions in solutions employed for calcification *in vitro,* both cyanide and fluoride ions exert a marked inhibitory effect. In the absence of magnesium ions, however, fluoride actually enhances the degree of mineralization, while cyanide appears to have neither a blocking nor an enhancing effect(1). Magnesium, in the amounts used, either exerted no measurable influence on the degree of mineralization or only a mild inhibition when cyanide or fluoride ions were not present. Recently Marks and his associates commented on the cyanide inhibition, stating that magnesium inhibition in itself accounts for the findings of cyanide inhibition(2). For this reason our experiments were carefully repeated and the data presented in Table IV of reference 1 were confirmed. It was shown at that time that only in the presence of magnesium ions added to the calcifying fluid will cyanide block the mineralizing process *in vitro.* The present work is concerned with an extension of the studies of cyanide and fluoride inhibition to calcifying media containing β-glycerophosphate as the source of phosphorus. In the course of these experiments it was found that cations other than magnesium, such as strontium, barium, and manganese, also cause cyanide to act as an inhibitor.

Robison and his co-workers(3,4) were first to observe inhibition of *in vitro* calcification by fluoride and cyanide ions in inorganic phosphate solution.‡ No cyanide effect was observed with β-glycerophosphate, although a block by fluoride was still evident. A reinvestigation of the problem by Gutman, *et al.* (5-7), served to confirm the earlier work of Robison, with one exception: neither cyanide nor fluoride interfered with calcification in mineral solutions containing phosphorus as β-glycerophosphate.‡ This point of discrepancy seemed to warrant further inquiry, particularly in view of the significance attached to the apparently dissimilar behavior of the ions in inorganic and organic phosphate medium(5-7).

Methods. A modified USP rachitogenic diet replaced the diet previously used(8). The rations, on which the Wistar strain albino rats were placed at the age of 22-24 days, consisted of 76% degermed corn meal, 20% wheat gluten, 3% $CaCO_3$, and 1% NaCl. The rachitic animals were sacrificed 17 to 23 days later. Slices of bone cartilage were removed from their tibiae and suspended in the designated solutions in 25 ml Erlenmeyer

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‡ In addition to calcium, phosphate, sodium, potassium, and bicarbonate, magnesium was included in the solutions of both Robison and Gutman to simulate its presence in serum. The critical role of magnesium in the inhibitory process has only recently been recognized(1).

TABLE I. Relative Rates of Calcification in β-Glycerophosphate and Inorganic Phosphate Media. (Ca^{++}) = 10 mg % = 2.5 mM.

Mg^{++} conc., mM/L	Incubation period, hr	Degree of calcification — Organic P conc. 3 mg %	4 mg %	5 mg %	6 mg %	10 mg %	Inorganic P conc., 5 mg %
0	5		2.0(4+)	2.5(4+)	2.8(4+)		2.6(4+)
1	5		1.3(3+)	1.6(3+)	1.9(4+)		1.7(3+)
1	6	1(+)		1.5(4+)		2.2(4+)	1.6(4+)
1	18			2.2(4+)		3.2(4+)	2.4(4+)
1	18		1.8(4+)	2.3(4+)	2.8(4+)		2.6(4+)

flasks from glass hooks affixed to the underside of paraffined cork stoppers. The upper level of the solution was kept within 4-5 mm of the bottom of the stopper. Incubations were conducted without shaking at 37°C and pH 7.3. Additional experimental details are given in Papers V(1) and IX(9) of this series. Each line of the accompanying tables represents results obtained from 2 to 4 rats. The source of β-glycerophosphate was the Mallinckrodt disodium salt, theoretical mol. wt. 315.15 based on a water content of 5.5 moles. Analysis of the compound revealed it to be free of inorganic P. Following digestion with a nitric-sulfuric acid mixture, the P assayed almost 6% in excess of theory (digestion blanks and inorganic P controls were run simultaneously). Stock solutions of β-glycerophosphate containing 1 mg organic P per ml were prepared by dissolving 0.960 g of the reagent in distilled water and diluting to 100 ml. The new mineral deposit formed *in vitro* was revealed by silver staining. The degree of calcification was graded as follows: no calcification, 0(0); maximum calcification, 4(++++); the length of the line or deposit is designated by the +'s and its thickness or width by the number preceding the parenthesis (10). Since the width of the deposit is often not uniform, we have adopted the procedure of assaying each quarter of the line (under 60 X magnification) and then taking the average. Agreement was generally obtained between the mean values for the 2 sides of a given slice, and also for the 2 or 3 slices from each tibia.

Results. In order to assess the effect of cyanide and fluoride ions on calcification in β-glycerophosphate medium relative to their action in inorganic phosphate, it was first necessary to determine what levels of organic and inorganic phosphate yield equivalent amounts of mineral deposit. No systematic effort to fix this relationship appears to have been made by the earlier workers. The reference system used here, as well as in our previous study(1), consisted of basal salt (70 mM NaCl, 5 mM KCl, and 22 mM $NaHCO_3$ per liter), 10 mg % (2.5 mM) Ca^{++}, and 5 mg % (1.61 mM) P as inorganic phosphate. The test solutions contained 3 to 10 mg % P as β-glycerophosphate but were otherwise identical in makeup. Incubations were conducted for 5 to 18 hours in the presence and absence of Mg^{++}. As contrasted with the mineral deposit acquired by the slices of cartilage in 5 mg % inorganic P, the degree of calcification in organic phosphate was found to be markedly decreased at 3 to 4 mg %, approximately equal at 5 to 6 mg %, and greater at higher concentrations (Table I). From the tabulated data it was ascertained that 5 mg % inorganic P is equivalent to a mean value of 5.5 mg % β-glycerophosphate P.

Having established that rachitic rat cartilage calcifies to about the same extent in solutions 10/0/5.5§ and 10/5/0,§ incubations were carried out in organic medium to determine the effect of 1 mM KCN. No cyanide inhibition was noted, even in the 5-hour period (Table II). *Inhibition was obtained* in the cyanide flasks, however, when the solution was supplemented with 1 mM Mg^{++}. At the concentrations of Mg^{++} and cyanide employed here, the block of calcification was usually complete in the first 5 hours, but

§ The symbol a/b/c is used to represent a mineral solution containing the basal components and: a, mg% Ca^{++}; b, mg% inorganic P; and c, mg% organic P (here, β-glycerophosphate).

TABLE II. Magnesium-Dependence of Cyanide Inhibition in β-Glycerophosphate Medium. (10/0/5.5)

Mg^{++} conc., mM/L	Cyanide conc., mM/L	Incubation period, hr	Degree of calcification: Test tissue	Control*
0	1	5	2.5(4+)	2.3(4+)
0	1	18	2.8(4+)	2.8(4+)
1	1	5	1 (1+)	1.8(4+)
1	1	18	1.9(2+)†	2.3(4+)

* No cyanide present in calcifying solution.
† Subsurface deposit.

rarely so after 18 hours of incubation. Similar observations have been recorded for the system 10/5/0(1).

Calcification in organic as well as inorganic (1) medium containing Mg^{++} was blocked in the presence of 10^{-4} to 10^{-3} molar fluoride (Table III), and more decisively than with cyanide ions. In contrast to the results obtained with cyanide, 10^{-4} molar fluoride proved to be inhibitory in the first 5 hours in the absence of added Mg^{++}, although calcification did occur within 18 hours. Less inhibition was noted at the 10^{-3} molar fluoride level after the first 5 hours, while in 18 hours the degree of calcification actually exceeded the control sections, but not markedly. Identical results were obtained in solution 10/5/0 containing 10^{-3} molar fluoride and no Mg^{++}. The less pronounced increase in deposition over the fluoride-free control obtained here in both organic and inorganic media, as compared to our previous experiments(1), may be a result of the change in the rat diet. The mineral deposit formed in fluoride solution could be visualized prior to silver staining as an opaque white band present below the epiphyseal plate. Microscopic examination following staining revealed a typical honeycomb structure which was restricted to the metaphysis and undoubtedly represents *bona fide* calcification(1).

It was of further interest to determine whether the Mg^{++}-linked inhibition by fluoride and cyanide could be overcome at higher phosphate levels, as appeared likely from the literature, and particularly whether an equivalent response would be evoked in inorganic phosphate and β-glycerophosphate media. As the P concentrations were raised above 8 mg % inorganic P and 10 mg % organic P, however, difficulty was encountered in attempting to equate organic to inorganic phosphate. In both cases extensive and often limiting deposits were obtained in which the areas could not be easily differentiated. For this reason a calcifying solution was sought which was sufficiently dilute to yield submaximal deposits, and yet sufficiently concentrated so as to partially repress the action of the inhibitors. Solution 10/0/10 appeared to be suitable and was chosen as the reference system (Table IV). Mg^{++} was included in all media in 1 mM concentration. Studies of the type recorded in Table I were then made in which the degrees of mineralization at 10/7/0, 10/7.5/0, 10/8/0, 10/9/0, and 10/0/10 were compared at 5 and 18 hours. Solution 10/0/10 proved to be approximately equivalent to 10/7.5/0-10/8/0.

Inhibition in the presence of 1 mM cyanide was found to decrease gradually between 10/5/0 and 10/7/0 and more rapidly above 10/7/0. Virtually no inhibition could be detected in solution 10/8/0 after 18 hours of incubation, as was the case with the equivalent organic solution 10/0/10 (Table IV).

Raising the P concentrations also tended to reverse the Mg^{++}-fluoride block in both organic and inorganic phosphate media, but not as readily as with Mg^{++}-cyanide. The degree of calcification in solutions 10/8/0 and 10/0/10 was found to be the same within the limits of error of assessing the poorly defined deposits. In these experiments, as in others involving inhibitors, the degree of inhibition

TABLE III. Magnesium-Dependence of Fluoride Inhibition in β-Glycerophosphate Medium. (10/0/5.5)

Mg^{++} conc., mM/L	Fluoride conc., mM/L	Incubation period, hr	Degree of calcification: Test tissue	Control*
0	.1	5	1.1(2+)	2.3(4+)
0	.1	18	2.6(4+)	2.8(4+)
0	1	5	2.5(3+)	2.3(4+)
0	1	18	2.9(4+)†	2.5(4+)†
1	.1	5	0 (0)	1.8(4+)
1	.1	18	0 (0)	2.3(4+)
1	1	5	0 (0)	1.8(4+)
1	1	18	1 (+)	3.1(4+)

* No fluoride present in calcifying solution.
† Identical results obtained with sol. 10/5/0.

TABLE IV. Relief of Cyanide and Fluoride Inhibition at Increased Inorganic and Organic Phosphate Levels. (Ca^{++}) = 2.5 mM, (Mg^{++}) = 1 mM.

Cyanide conc., mM/L	Fluoride conc., mM/L	Incubation time, hr	Inorganic phosphate: Conc., mg % P	Inorganic phosphate: Degree of calcification, Test tissue	Inorganic phosphate: Degree of calcification, Control*	β-Glycerophosphate: Conc., mg % P	β-Glycerophosphate: Degree of calcification, Test tissue	β-Glycerophosphate: Degree of calcification, Control*
1	0	18	7	1.9(3+)	2.6(4+)	10	2.3(4+)	2.7(4+)
1	0	18	8	3.6(4+)	3.7(4+)	10	3.6(4+)	3.7(4+)
0	.1	18	8	~3 (4+)†	3.2(4+)	10	~3 (4+)†	3.3(4+)

* Calcifying solutions devoid of cyanide and fluoride.

† Deposit did not possess the depth or clarity of the control section. Virtually no calcification was noted in a 5-hr incubation period.

became most pronounced with cartilage slices from rats maintained on the rachitogenic diet for more than three weeks.

Role of cations in the inhibition phenomenon. As indicated in this and earlier papers (1,11), cyanide, unlike fluoride, has no influence *per se* on *in vitro* calcification. The common physiological cation Mg^{++} readily mediates the cyanide effect, while the monovalent Na^{+} and K^{+} ions present in calcifying solution appear to have no action. To determine the specificity of the phenomenon, other ions were also tested without regard to their possible biological distribution. The results are given in Table V. Like Mg^{++}, the ions Sr^{++}, Ba^{++}, and Mn^{++} were found to be inhibitory in conjunction with cyanide, but not significantly in its absence at the designated concentrations (incubation period, 18 hours). However, these cations blocked calcification when their concentrations were increased severalfold. Of interest in this connection is the remarkably inhibitory nature of Mn^{++}, which was apparent at a concentration of 0.05 mM per liter = 1/50 of the calcium content of the calcifying solution.

Discussion. It is evident from these studies that cyanide and fluoride ions, in the presence of *magnesium* ions added to the calcifying solutions, inhibit calcification *in vitro* not only with inorganic phosphate(1), but also with β-glycerophosphate as the sole source of phosphate. In the absence of *added magnesium,* cyanide does not inhibit, while 10^{-4} molar fluoride exerts an inhibitory effect in the first few hours which disappears later. This initial inhibition may be due to magnesium originally present in the bone cartilage which diffuses out later. When the concentration of fluoride is 10^{-3} molar the degree of mineralization is actually enhanced, while in the presence of magnesium mineralization is blocked. It must be pointed out in reference to the comments of Marks, *et al.*(2), that magnesium ions in the concentration employed either had no effect or were only mildly inhibitory. The comparison of fluoride or cyanide with magnesium was always made against control bone sections in solutions containing the same concentrations of magnesium.

The nature of our findings with fluoride and cyanide ions in β-glycerophosphate medium requires a reexamination of current theories of calcification. It has been suggested by Gutman and coworkers(5-7) that calcification may proceed along either of 2 completely discrete pathways, depending on the form in which phosphorus is supplied to the mineral solution. The first is assumed to involve phosphorylative glycogenolysis and requires phosphorus in the form of inorganic phosphate or a phosphorylated glycolytic intermediate. The second pathway is thought to involve hydrolysis by bone phosphatase of its typical substrates not occurring in the scheme of glycolysis, such as β-glycerophosphate or phenyl phosphate, with the local liberation of excess inorganic phosphate and its simultaneous deposition as bone salt. The conclusion that inorganic phosphate released by phosphatase action does not enter the glycolytic cycle was based on the failure to demonstrate fluoride inhibition of this second mechanism. Since our findings now indicate that the nature of the action of fluoride or cyanide ions *is* the same in β-glycerophosphate as in inorganic phosphate under comparable experimental conditions, there would appear to be no justification at this time for assuming two such wholly discrete pathways. Indeed,

TABLE V. Effect of Cyanide on Calcification in the Presence of Divalent Cations Other than Magnesium. (Incubation period, 18 hours.)

Cation	Conc., mM/L	Cyanide conc., mM/L	Degree of calcification: 10/5/0 Test tissue	10/5/0 Control*	10/0/5.5 Test tissue	10/0/5.5 Control*
Sr^{++}	1	1	0 (0)	1.6(4+)		
Ba^{++}	.1	1	1.3 (+)	2 (4+)	1(+)	1.8(4+)
	.5	0	0 (0)	2 (4+)†		
Mn^{++}	.01	1	1 (2+)	1.9(4+)		
	.01	1			0 (0)	1.8(4+)
	.05	0	0 (0)	1.8(4+)†		
Co^{++}	.02	1	2 (4+)	2 (4+)		
Ni^{++}	.02	1	2 (4+)	2 (4+)		

* The control calcifying solutions were devoid of cyanide but contained the specified cations, unless otherwise indicated.

† Control calcifying solution devoid of both cyanide and the specified cation.

from recent evidence(12), taken in conjunction with the present paper, it would appear to be doubtful whether any pathway has as yet been decisively established.

The tendency for high concentrations of phosphate to counter or obscure the inhibition by magnesium-fluoride and, particularly, magnesium-cyanide, the weaker couple, is a likely explanation for the apparent discrepancies that have arisen. The indifferent effect of cyanide in organic mineralizing solutions noted in earlier studies may be attributed to the excessive amounts of glycerophosphate (6) or glycerophosphate plus inorganic phosphate(3,4) employed as compared to the control inorganic medium. That the more intensive block by fluoride may be detected in glycerophosphate medium at as high a concentration as 10 mg % P was originally reported by Robison and Rosenheim(4), but subsequently overlooked in later studies in which still higher concentrations of phosphate were employed(6).

It is of considerable interest that the several ions found to mediate cyanide inhibition, including magnesium, strontium, barium, and manganese, are themselves inhibitory at higher concentrations. Possibly they act by interfering with adsorption or diffusion phenomena at the tissue surface, or with growth of the bone lattice. If this is the case, cyanide ion may serve to complex the metal ion at its site of action and increase its inhibitory activity by preventing its release.

Summary. 1. The influence of several ions on *in vitro* calcification in β-glycerophosphate medium has been compared to their effects in inorganic phosphate solution at two phosphate levels which yield comparable degrees of calcification, *viz.*, 10/5/0-10/0/5.5 and 10/8/0-10/0/10.§ Cyanide ion did not interfere in the absence of magnesium. When magnesium was present there was a partial block of calcification at the lower organic phosphate level equivalent to that obtained in the corresponding inorganic solution. At the higher phosphorus levels, 10/8/0-10/0/10, virtually no inhibition by the magnesium-cyanide couple was evident. 2. Calcification in the dilute glycerophosphate solution (10/0/5.5) was blocked by fluoride in the presence of magnesium. In the absence of magnesium, inhibition by 10^{-4} molar fluoride was noted during the first few hours, but not evident on prolonged incubation. Mineral deposition with 10^{-3} molar fluoride in the absence of magnesium exceeded that obtained with the fluoride-free control. Essentially similar results were presented earlier for the equivalent inorganic mineral solution, 10/5/0(1). At higher inorganic and organic phosphorus levels the fluoride block in the presence of magnesium was partially overcome. 3. The data indicate a striking similarity in the response of the calcifying mechanism in both inorganic and β-glycerophosphate media to the action of fluoride or cyanide ions in the presence and absence of magnesium. It therefore appears

questionable whether one may, at this time, associate the calcification process in inorganic phosphate and glycerophosphate solutions with two completely discrete mechanisms. 4. Strontium, barium, and manganous ions share with magnesium the property of mediating the cyanide inhibition of calcification.

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Effects of a Combined Deficiency of Vitamins E and B_6 on Blood Picture of Rat.* (20854)

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One sign of acute vit. E deficiency in monkeys and rabbits is leucocytosis(1,2). There is a marked increase in the numbers of circulating granulocytes with little change in the numbers of blood lymphocytes(1,2). Vitamin B_6 is also concerned in hemopoiesis. It has been shown that B_6-deficient dogs(3) and pigs(4) develop a microcytic anemia. Weir *et al.*(5) reported that the injection of a vit. B_6 antagonist (desoxypyridoxine) into mice resulted in a decrease in circulating lymphocytes and an increase in granulocytes. These changes could be reversed by the administration of pyridoxine. Quite recently Vilter *et al.*(6) reported the effects of administering desoxypyridoxine to man. They observed an absolute lymphopenia with a tendency toward polymorphonuclear leucocytosis. In contrast Wintrobe *et al.*(4) observed no significant change in leucocytes as a result of vit. B_6 deficiency in pigs.

In general, the leucocyte changes which have been observed in vit. B_6 deficiency resemble those of vit. E deficiency. There is some biochemical data which suggest a relationship between vit. E and vit. B_6 in metabolism(7,8). For these reasons a study was made of the effect of a combined deficiency of vit. E and vit. B_6 on the hemogram of the rat.

Methods. Weanling littermate Sprague-Dawley rats of both sexes were fed a basal

TABLE I. Weight Changes and Food Consumption of Rats Fed the Experimental Diets.

Diet	No. of rats	Mean initial wt, g	Mean final wt, g	Mean daily food intake, g
Basal	9	50	102	4.6
" + E	7	49	106	5.4
" + B_6	7	47	194	8.6
" + B_6 + E	7	48	223	8.3

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TABLE II. Effects of Vit. E and B_6 on Peripheral Leucocytes of Rats (Leucocytes Reported as Thousands/μl).

Diet	Total leucocytes	Lymphocytes	Monocytes	Neutrophils	Eosinophils
Basal	18.4	6.9	.6	10.5	.4
" + E	8.5	5.9	.1	2.4	.1
" + B_6	10.0	7.2	.4	2.2	.2
" + B_6 + E	10.8	8.8	.4	1.5	.1

diet consisting of casein, 18.6 g; sucrose, 67.4 g; lard, 8.0 g; cod liver oil, 2.0 g; salt mix (*J. Nutrition,* v14, 273), 4.0 g: choline chloride .1 g; inositol, 10 mg; riboflavin, .8 mg; thiamin HCl, .5 mg; Ca pantothenate, 2 mg; nicotinic acid, 2 mg; and menadione, .44 mg. This diet is deficient in vit. E and B_6. One group of rats was given this diet without supplement. Three other groups of rats were given the basal diet supplemented with pyridoxine, α-tocopheryl acetate, and the combination of the two, respectively. Pyridoxine was mixed with the diet at a level of 10 mg per kilo. Tocopherol was given orally 2 times weekly. The animals were given 2 mg of α-tocopherol acetate per dose until they reached a body weight of 100 g at which time the dose was increased to 4 mg. Food and water were given *ad libitum* and food intake records were kept. The rats were fed these diets for 105 days. Complete blood counts on tail blood were made at frequent intervals, using standard hematological technics.

Results. The effects of the supplements on weight gain and food intake are given in Table I. Vit. B_6 markedly increased growth and food consumption. Supplementation of the basal diet or the vit. B_6-containing diet with vit. E had no significant effect on growth or food intake.

Erythrocyte counts and hemoglobin, and hematocrit determinations were made on tail blood from all rats at frequent intervals. The results were all within the normal limits for the rat and the data are not given.

The leucocyte data given in Table II were obtained between the 95th and 105th experimental days. The values are the mean of 2 to 3 bleedings for each rat in the various groups. Monocytes and eosinophils were present in insignificant numbers in all groups. Animals receiving the diets without vit. B_6 exhibited slightly lower lymphocyte counts than did the vit. B_6-supplemented groups. This difference in number of lymphocytes was found to be statistically significant when the data were analyzed by the analysis of variance method ($P < 0.05$). It must be realized that animals in the vit. B_6-deficient groups consumed less food than did the B_6-supplemented animals and inanition has been shown to result in a reduction in the numbers of peripheral lymphocytes of rats(9).

The most striking effect observed was the great increase in circulating neutrophils in rats deficient in both vit. E and B_6. The numbers of peripheral neutrophils of animals in this group were elevated 7-fold when compared with control animals receiving both vitamins. Supplementation of the basal diet with either vit. E or B_6 markedly reduced the numbers of neutrophils. The combination of the 2 vitamins reduced neutrophils only slightly more than either of them separately.

In Fig. 1 neutrophil counts are plotted as a function of time on the various diets. The increase in number of neutrophils of rats fed the basal diet began about the 50th day of feeding and continued rapidly thereafter.

Discussion. The most striking finding in these experiments was the observation that rats deficient in both vit. E and B_6 exhibit a marked granulocytosis and that this condition is prevented by supplementing the diet with either vit. E or B_6. This indicates that these 2 vitamins are closely related in some metabolic mechanisms. Extensive biochemical studies were made on these animals and will be reported in detail elsewhere but it is appropriate to mention here that creatine excretion followed a pattern quite similar to that of the peripheral neutrophils. Animals receiving the basal diet exhibited an increased creatinuria beginning about the 50th experi-

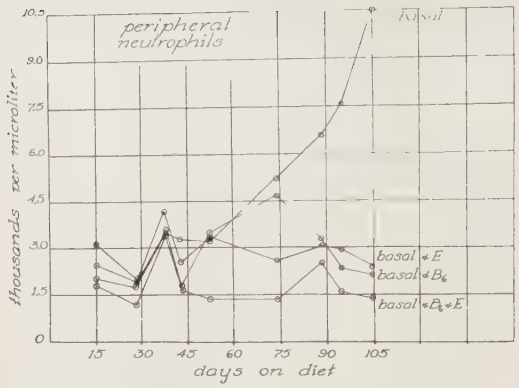

FIG. 1. Effects of vit. E and B_6 on peripheral neutrophils of rats.

mental day. This was approximately the same time the increased neutrophil levels became apparent. Creatinuria serves as a good indication of the beginning of nutritional muscular dystrophy in rabbits(10). Thus, rats deficient in both vit. E and B_6 behaved like rabbits deficient in vit. E, and granulocytosis may be a result of the metabolic alterations which instigate nutritional muscular dystrophy.

Summary. Sprague-Dawley rats deficient in both vit. E and B_6 exhibited a greatly increased peripheral neutrophil count. Supplementation of the diet with either of these vitamins prevented this increase. The B_6-deficient rats exhibited a slight lymphopenia which was not affected by vit. E. None of the animals developed anemia.

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Identification and Quantitative Determination of Serotonin (5-hydroxytryptamine) in Blood Platelets.*† (20855)

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The vasoconstrictor substance in blood serum was identified by Rapport and his coworkers as 5-hydroxytryptamine (serotonin) (1). Serotonin is not present in plasma(2), but appears in serum when clotting occurs in the presence of platelets(3,4). The fact that not only coagulation but also agitation, aging, freezing or exposure to ultrasonic frequencies cause the appearance of vasoconstrictor activity in platelet-rich plasma indicates that the clotting process *per se* is of no importance in the evolution of serotonin and suggests that the material is simply released from the platelets by these procedures. The occurrence of smooth muscle-stimulating material in crude platelet extracts supports this suggestion. In preliminary experiments, paper chromatograms of dog and rabbit platelet extracts were found to show spots which corresponded to 5-hydroxytryptamine(5). In the absence of information on the reliability of paper chro-

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matography for identification of this class of compounds, it seemed advisable to establish the identity of the material isolated by chromatography of platelet extracts by the simultaneous application of ultraviolet absorption measurements, colorimetry, and pharmacological assay. These experiments also permitted the determination of the quantity of serotonin in platelets and the examination of these cells for related substances.

Methods. The kindness of Dr. Seegers and his colleagues in making available to us dried bovine platelets was of immeasurable help. The platelets were separated from oxalated blood by differential centrifugation, washed once with about 3 volumes of oxalated saline, dried from the frozen state and sent to us(6). The properties of the platelet extracts were compared with those of standard serotonin solution (500 μg base/ml) prepared from synthetic 5-hydroxytryptamine creatinine sulphate.§ The standard, which was stable for days when kept in the icebox or freezer, was suitably diluted with saline before use. *Vasoconstrictor assays* were performed by a modification of the method of Page and Green(7) on the isolated perfused rabbit ear, by measuring the decrease in outflow of the perfusate after the injection of 0.1 ml into the arterial supply(8). Activity was also estimated from the contractions of the rat uterus isolated 18 to 24 hours after the injection of 10 μg/100 g of diethylstilbestrol. The *Folin-Ciocalteu reaction* was carried out as follows: To the sample in 2 ml of water, 0.5 ml of Folin Reagent (phosphotungstic acid) was added, followed by 1.5 ml of 15% aqueous Na_2CO_3. Two ml of water at 30°C were then added and the color read after 20 minutes in the Klett-Summerson colorimeter with a 66 filter.

Results. In preliminary experiments, 5 mg of dried bovine platelets were suspended in water (saline was equally effective) and refrigerated overnight. A considerable quantity of insoluble material remained. The activity of the supernatant as tested on the rabbit ear averaged 2.3 μg/mg dry platelets and was stable for at least a week in the refrigerator.

§ Kindly supplied by Dr. M. E. Speeter of the Upjohn Co.

To determine whether appreciable amounts of vasoconstrictor material remained in the insoluble residue, two experiments were performed in which the suspension of platelet material was subjected to ultrasonic vibrations for 5 to 15 minutes, producing a slightly opalescent solution. The pharmacological activity was not increased by this treatment, indicating that essentially all of the active material was obtained by overnight extraction.

For the chemical determinations, the extract was purified as follows. 51.8 mg of dry bovine platelets were suspended in 2.0 ml of water + 0.5 ml of acetone and placed in the refrigerator overnight. Then 38 ml of cold acetone were added. After standing in the refrigerator for 2 hours, the mixture was centrifuged in the cold at 900 x g for 30 minutes. The clear supernatant was removed and evaporated to dryness *in vacuo* with the aid of butanol and acetone. The residue was extracted with 5 ml of 95% aqueous acetone and the extract again evaporated to dryness *in vacuo*. The dry residue was taken up in 2 ml of water. This produced a flocculation of some lipid material. The mixture was centrifuged for 12 minutes at 20,000 x g. The supernatant solution (1.91 ml) was evaporated to dryness and taken up in 0.2 ml of 90% acetone. This solution was immediately placed on Whatman No. 1 paper in 5 individual spots, each representing 20 applications of 0.005 ml. Two control spots of 35 μg serotonin were applied to the same paper. Development was carried out by descending irrigation employing butanol saturated with N HCl(9) for 16 hours at 25°C. The solvent front had moved 30 cm. After drying the paper in air, one control and one extract strip were sprayed with p-dimethylaminobenzaldehyde(1) and dried at 105°C. Both showed a single spot with Rf = 0.15. The corresponding portions of the remaining four extract strips were combined and eluted with 4.5 ml of water at 37°C for 2 hours with shaking. The remaining control spot was eluted with 3.5 ml of water. Ultraviolet absorption spectra of these extracts are shown in Fig. 1. The deviations of the platelet extract curve from that of serotonin (slightly

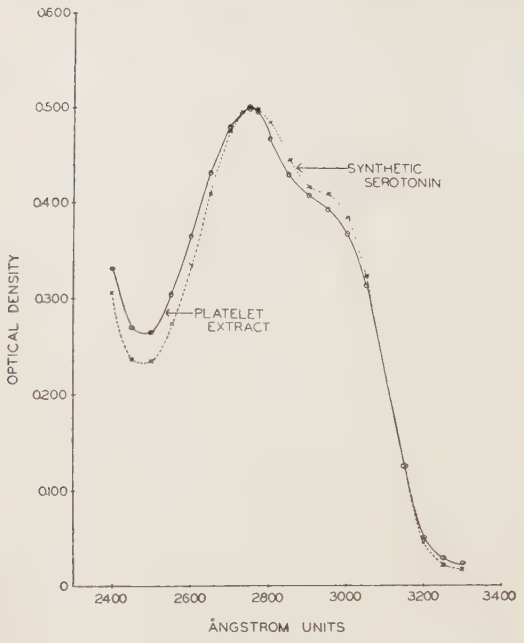

FIG. 1. Ultraviolet absorption spectrum of the substances eluted from the paper chromatogram: ⊙——⊙ Bovine platelet extract, pH 2.4. ×---× Synthetic serotonin eluted from the chromatogram $\left(\times\text{---}\frac{502}{300}\right)$, pH 2.5.

increased absorption below 2700 Å and above 3200 Å, and slightly decreased absorption between 2800 and 3000 Å) indicate that a small amount of deterioration occurred during the manipulation of the material. These changes are exaggerated when elution of the developed chromatogram is delayed. More extensive decomposition produced a shift of the maximum to as low as 2700 Å with a loss in pharmacological activity of about 50%. The eluted extract had a 5-hydroxytryptamine content of 14.2 μg/ml calculated from the O.D. at 2750 Å ($\epsilon = 6200$), 14.2 μg/ml by Folin-Ciocalteu reaction, and 15 μg/ml by pharmacological assay on the rabbit ear and rat uterus. There is therefore no question that the material corresponds perfectly in properties to 5-hydroxytryptamine. The recovery of material, taking into account the volume losses during extraction and a recovery on elution from the paper chromatogram of 87%, corresponds to 98 μg of the base (1.9 μg/mg dry platelets), or 75% of that found in the crude platelet extract by pharmacological assay. In view of the instability of serotonin, this represents an excellent recovery.

No other indolic or phenolic substance was present on the paper chromatograms as determined by spraying with dimethylaminobenzaldehyde or with diazotized p-nitroaniline (9). This observation suggests that precursors of 5-hydroxytryptamine such as 5-hydroxytryptophane(10) do not occur in significant amounts in the platelet and must therefore be looked for in the megakaryocytes. The high percentage of the pharmacological activity of the crude extract which can be attributed to 5-hydroxytryptamine indicates that no other potent substance active on the vasculature of the rabbit ear or on the rat uterus is present in bovine platelets.

The platelet count of the slowly-centrifuged platelet-rich plasma employed as the starting material is maximally 1.1 x 10^6/mm^3 (estimated from the average values for bovine whole blood platelet count (684,000/mm^3) and hematocrit (40%)(11). Since about 4 g of dry platelets are obtained from 5 liters of this plasma(6), and since our assay has established that serotonin represents 2.3 μg/mg of dry platelets, it can be calculated that each platelet contains at least 1.7 x 10^{-9} μg of serotonin. The actual figure may well be several times higher because the yield of platelets in preparing the dry material is surely less than 100%. If all of the serotonin in beef serum (1.5 μg/ml)(12) is assumed to come from the platelets, it can be calculated that the amount of serotonin per platelet is 1.4 x 10^{-9} μg. This remarkably good agreement demonstrates that serum serotonin is entirely derived from the platelets, and that there is no reason to believe that plasma constituents play a role in its evolution other than by contributing to the breakdown of platelets during clotting.

Summary. Serotonin (5-hydroxytryptamine) was identified in bovine platelets by the coincidence of its ultraviolet absorption spectrum with that of synthetic serotonin, and by the agreement of analyses based on ultraviolet absorption, Folin-Ciocalteu reaction and pharmacological assay using the rabbit ear

and rat uterus. An average of 2.3 μg/mg of dry platelets was found, equivalent to at least 1.7 x 10^{-9} μg/platelet. This is more than sufficient to account for all of the serotonin found in bovine serum. No other indolic or phenolic substances were found in the platelet extracts.

Addendum. After this paper was in press, we learned that M. Bracco and P. C. Curti (*Haematologica,* 1953, v37, 721) had identified 5-hydroxytryptamine in rabbit and sheep platelets by similar methods.

The authors wish to express their appreciation to Miss Jennie Borrelli for her capable assistance.

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Experimental Aortic Disease in Albino Rats Following Renal Operations.* (20856)

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Striking changes in the aortae of male albino rats were observed at autopsies performed from one to 13 months following renal operations previously shown(1-4) to induce hypertension and renal disease. The aortae were increased in diameter and length, moderately tortuous in some instances and showed complete or incomplete ring-like bands of calcification giving them an appearance rather like trachea. The rats at the time of autopsy were from four to sixteen months old. Table I gives the incidence of grossly visible lesions in experimental and control rats. Since mild degrees of the aortic lesion were observed in one male and one female rat of a group of untreated animals previously set aside to age relevant data from this group have been included in the table.

Fig. 1 shows one of the more severely affected aortas. The bend pictured was present in the vessel during life so that it was appreciably displaced from the vertebral column. The lumpy, paler zones along the course of the aorta represent foci of medial calcification. Fig. 2 is a roentgenogram of this vessel. Fig. 3 is a magnified roentgenographic view of the same vessel compared with a control aorta from a rat of the same age. These diseased aortas appear very large in comparison with normal aortas after removal. Partly this is due to a genuine increase in size, attested to by curvature and beginning tortuosity, partly to failure of the diseased aorta to contract after removal.

Microscopic studies. Sections were stained with hematoxylin and eosin, by the van Gieson-Weigert and the von Kossa technics. The earliest stage of the lesion appeared to be associated with a deposition of finely granular basophilic material, identified as calcium salt by the von Kossa stain, in the spaces between the concentrically arranged elastic lamellae. Whether this deposition oc-

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TABLE I.

Procedure*	Age, mo (operation)	Age, mo (autopsy)	No. rats	Calcific aortic disease
¾ nephrectomy, ♂	3	4–16	33	12
Renal constriction, ♂	2–3	4–13	11	6
Controls	2–3	10–14	22	0
Aging males	—	16–30	25	1
" females	—	15–30	22	1

* In the three-quarters nephrectomized group one kidney and half the other were removed. In the renal constriction group one kidney was removed and the other constricted with a figure of 8 ligature. The kidneys of the controls were exposed and briefly handled.

curred in the ground substance or in muscle cells was not determined. At a later stage the elastic lamellae sometimes contained calcium salts. With more severe degrees of the lesion there was destruction of muscle and elastic tissue with replacement by lumpy basophilic calcium-containing material. Ossification was not seen but a large mass of hyaline cartilage was found in one severely involved aorta. The lesions in the 2 aging rats, not operated on, were of the same character. Both of these rats were over 2 years of age and had severe renal disease. No changes in the intima were visible. Calcification of the kidneys, with involvement of the basement membranes and cells of the proximal convoluted tubules, was seen in most of the rats with aortic disease though it was absent in a

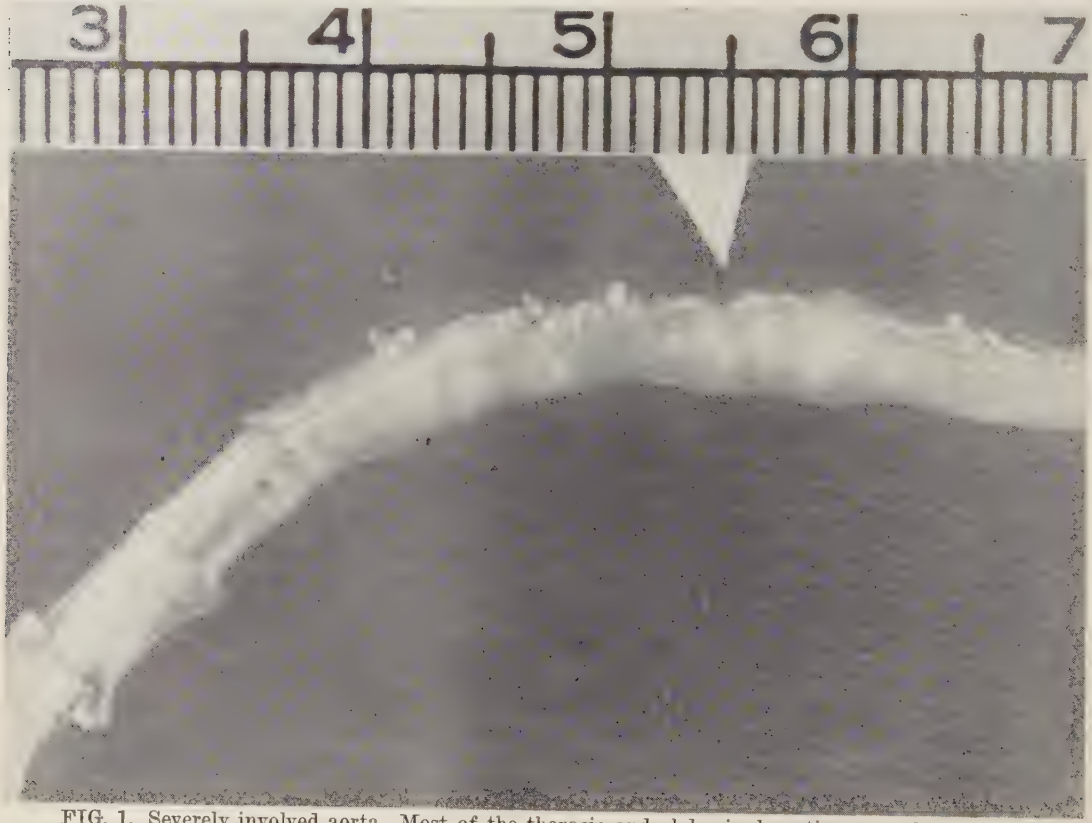

FIG. 1. Severely involved aorta. Most of the thoracic and abdominal portions are shown. The pale nodulations represent sites of medial calcification. 3×.

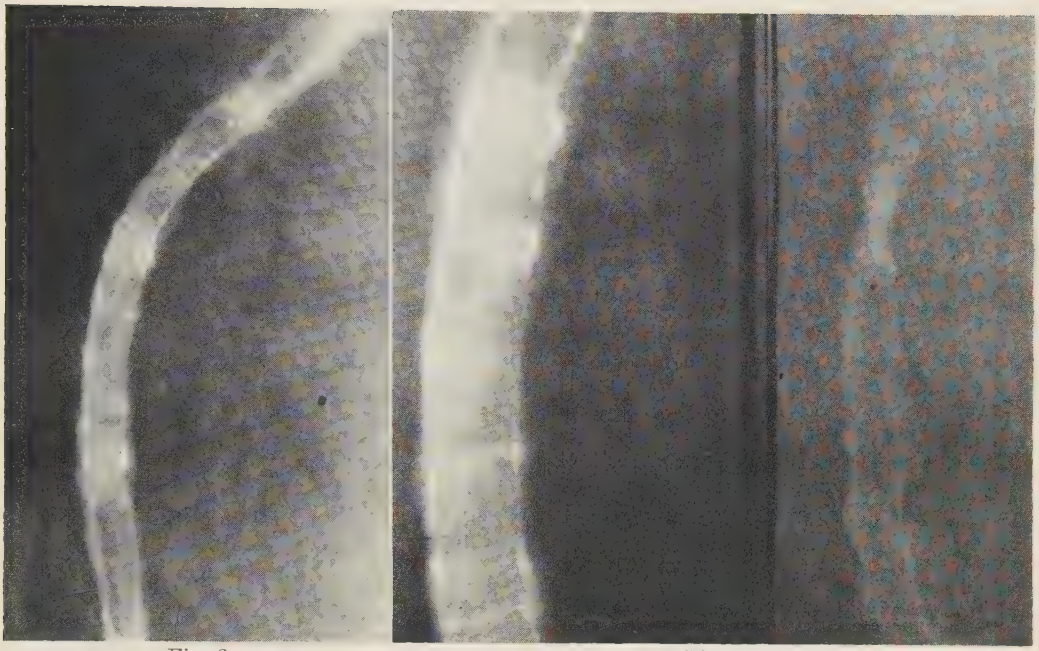

Fig. 2 Fig. 3

FIG. 2. Roentgenogram of aorta shown in Fig. 1. Ring-like and nodular calcification of wall. 1.25×.

FIG. 3. Roentgenogram of aorta shown in Fig. 1 and 2 compared with a control aorta from a rat of the same age. The opacity at the point of constriction of the control aorta is due to a drop of fluid. 3×.

few. All of the rats, however, had advanced renal disease of the type previously described (1,3) with dilatation of tubules and destructive changes in the glomeruli.

Discussion. With regard to the pathogenesis of the aortic lesions several possibilities present themselves. It has been shown by Pappenheimer(1) that albino rats, after three-quarters nephrectomy and the development of progressive destructive renal lesions, go on to develop secondary hyperparathyroidism in association with renal failure. Pappenheimer did not describe vascular lesions in his rats. In secondary as well as primary hyperparathyroidism the increased calcium-phosphorus ion product would be expected to favor metastatic calcification. Richardson(5), in a report of a case of chronic renal disease with secondary parathyroid hyperplasia and metastatic calcification, states that the common sites of calcification in chronic renal disease are the lungs, kidneys, subcutaneous tissue, heart and muscular arteries, while the stomach, contrary to the usual belief, is not usually involved. Richards(6) reported a case of renal disease with secondary hyperparathyroidism in which calcification of blood vessels was very severe. The 44-year-old female subject of his report had severe hypertension and roentgenological evidence of calcification of the meningeal, basilar, common iliac, popliteal, radial and digital arteries. In addition there was advanced atheroma of the aorta plus medial degeneration and calcification. Parathyroid hormone can produce medial calcific lesions in the aortas of dogs, rats, and rabbits together with calcification of the lungs, kidneys, stomach and smaller blood-vessels, and, according to Hueper, there is prior degeneration of the muscle cells of the media with temporary preservation of the elastic elements(7).

The severity of the lesions in the rats of this series suggests that the aortas were predisposed to damage by some structural or chemical alteration not yet understood. It may be related to hypertension. All of the animals had hypertrophied hearts and at one

time or another had shown elevated tail systolic blood pressures. It appears that in addition to the destructive calcific lesion there is a separate histological process of adaptation to high blood pressure going on. Evidence for this is the hypertrophy of elastic tissue elements, with the development of short cross-over bands between concentric elastic lamellae, seen in aortas which did not show the calcific lesions as well as those which did. Development of these changes was associated with alterations of the shape of the stress-strain curve when the aortas were subjected to longitudinal stretch.

Summary. The development and incidence of medial calcific disease of the aorta following operations on the kidneys of male albino rats is described.

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Synthesis of Amino Nitrogen from Ammonia in *Hymenolepis diminuta.** (20857)

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The rat tapeworm, *Hymenolepis diminuta,* is essentially independent of the protein in the diet of its host. Chandler(1) showed that no significant change in worm size, weight, or egg production could be induced under conditions of protein deprivation. Under such conditions it is quite probable that a considerable part of the amino nitrogen needed for growth and egg production comes directly from the host in the form of amino acids diffused from the intestinal mucosa. There is, however, the possibility that other sources of nitrogen contribute to these synthetic mechanisms. An investigation of the ammonia content of the rat intestine showed that a considerable amount was consistently present. The function of ammonia in intermediary protein metabolism is not very well understood; however, several workers have shown that it can contribute to the synthesis of certain amino acids and related substances(2,3). On the basis of these studies on mammalian tissues an investigation of the role of ammonia in the protein metabolism of *H. diminuta* was begun.

Materials and methods. Mature tapeworms, raised in male albino rats of the Sprague-Dawley strain, were infected with *H. diminuta* cysticercoids according to the technic of Chandler(1). The worms were removed from the intestine by flushing with isotonic KCl. They were thoroughly washed and were incubated singly in a Krebs-Ringer-Phosphate (KRP) medium modified by the addition of 0.004 M $(NH_4)_2CO_3$ and 0.1 M sodium pyruvate, 0.1 M sodium alpha-ketoglutarate, 0.1 M sodium oxalacetate, 0.1 M sodium succinate, or 5% glucose. As a means of blocking the tricarboxylic cycle, M/30 sodium malonate was included in several of the experiments in which glucose served as the substrate. The incubations were carried out in air at 38°C. At the end of 90 minutes the worms were removed, weighed, homogenized, and the free amino acids extracted for chromatographic resolution. The paper chromatograms were run on Whatman No. 4 with phenol as the

* This investigation was supported in part by research grant from The National Institutes of Health, Public Health Service.

TABLE I. Amino Nitrogen in mg Produced by 1 g (Dry Weight) *H. diminuta*/Hour.

	Incubation media	No. of animals	Amino N.
A	KRP	12	.13
B	+ $(NH_4)_2CO_3$	12	1.4
C	+ alpha ketoglutarate	12	4.8
D	+ pyruvate	12	6.3
E	+ oxalacetate	12	7.4
F	+ glucose	8	2.8
G	+ succinate	10	2.4
H	+ glucose + malonate	9	2.6

solvent. Development was with ninhydrin. No attempt was made at this time to estimate quantitatively the individual amino acids. The media were analyzed for free amino nitrogen by the photometric method of Frame, Russell, and Wilhelmi(4). These results were checked by comparison with the ninhydrin method of Van Slyke, MacFayden, and Hamilton(5). *Controls* were run in KRP from which the tissue or substrates were omitted. No amino nitrogen was found when the worms were omitted but a small amount appeared in those tubes in which the worms alone were present. This was assumed to be the result of a diffusion from the worm itself and this amount was subsequently subtracted from the value obtained from the experimental incubation to give a corrected figure. A second type of control involved the use of liver slices as a check on certain of the worm experiments. The results of these incubations were chromatographically analyzed by the procedures described above.

Results. The incubation of *H. diminuta* in the presence of NH_4 ions and various substances associated with glucose metabolism resulted in marked increases in amino nitrogen (Table I). With media containing the salts of alpha-ketoglutaric acid, pyruvic acid, or oxalacetic acid the mean amino nitrogen produced, in mg per g dry weight of worm, was 4.8, 6.3, and 7.4, respectively. It is noteworthy that these results parallel, in general, the results obtained on transaminase activities in the rat tapeworm with regard to the substrate effectiveness of these three keto acids(6). A small amount of amino nitrogen was produced when the NH_4 ions alone were included. This would seem to indicate that ketoacids, comparable with those associated in mammalian tissues with the tricarboxylic cycle, were present in the tapeworm, and that they were capable of acting as acceptors of the ammonia nitrogen. The chromatograms of the worms from this experiment bear out this point (Col. B, Fig. 1); they show that amino acids directly related to some of these keto acids were greatly increased in amount following the incubation with ammonia, *e.g.*, alanine and glutamic acid.

The ability of the tapeworm to utilize glucose as a substrate for amino nitrogen production is shown in F and H of Table I and Fig. 1. The mechanism of this activity undoubtedly involves a degradation of the glucose to intermediary keto acids and the amination of these. Malonate by its blocking of the succinic dehydrogenase system apparently prevented an optimum synthesis of amino nitrogen from glucose and ammonia. A further demonstration of the operation of the tricarboxylic cycle in *H. diminuta* was observed when succinate was included in the reaction medium. In this case significant increases in alanine as well as glutamic acid were observed.

The fate of the ammonia nitrogen in the tapeworm is demonstrated in Fig. 1. It is of interest to note that the major product of incubation, regardless of substrate, was alanine. This may be considered to be an indication of the essentially anaerobic existence of *H. diminuta*. Chromatograms of liver slices following incubation in media fortified by the inclusion of ammonia ions and/or alpha-ketoglutaric acid or pyruvic acid are given for comparison. It should be noted that the marked tendency to concentrate the free amino nitrogen in alanine did not exist here. In contrast to the tapeworm, the glutamic acid-alpha-ketoglutaric acid system appeared

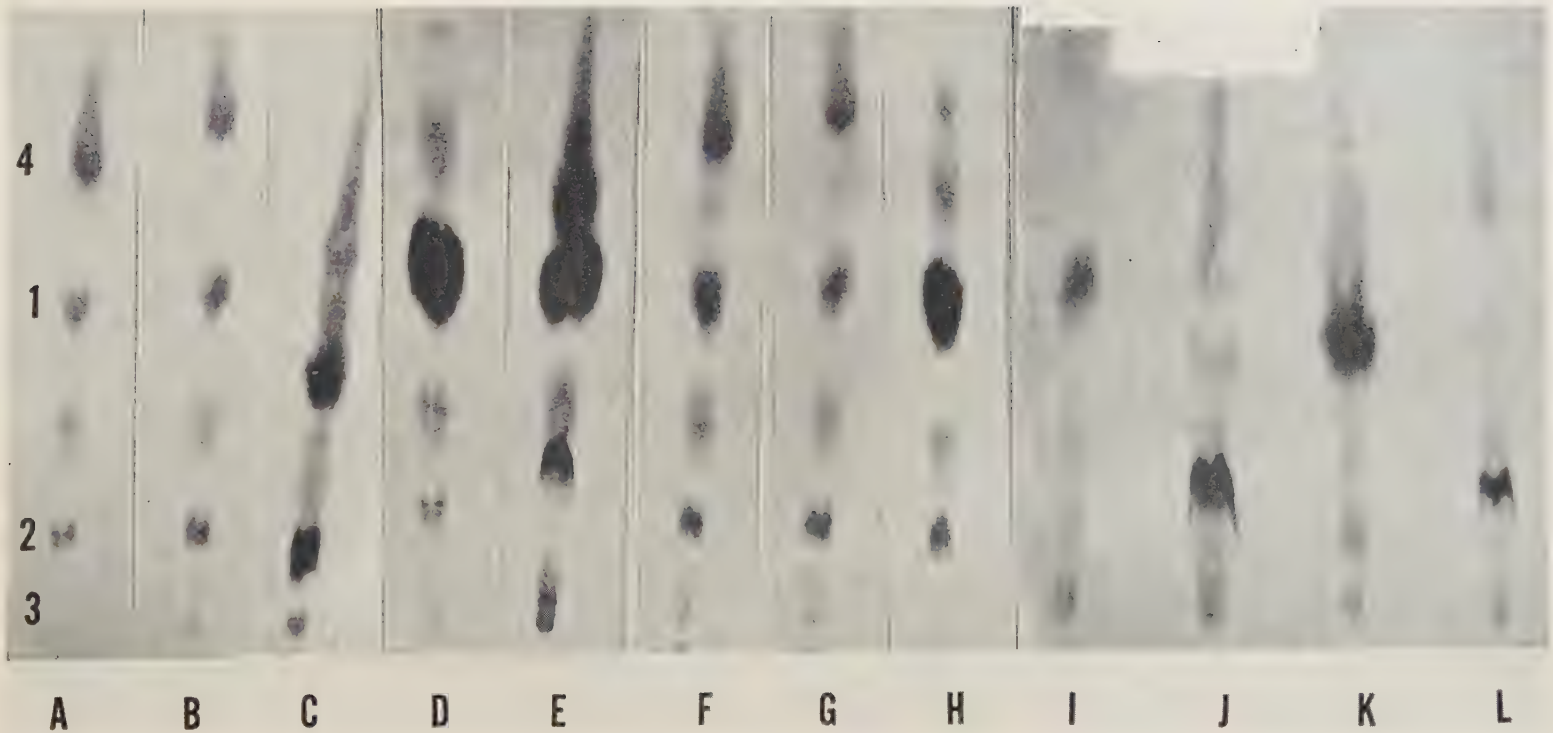

FIG. 1. Paper chromatograms showing amino acids resulting from incubation of *H. diminuta* (A-H) and rat liver slices (I-L) in the following media:

A	KRP	G	KRP + $(NH_4)_2CO_3$ + sodium succinate
B	+ $(NH_4)_2CO_3$	H	+ glucose + sodium malonate
C	+ sodium alpha-ketoglutarate	I	sodium pyruvate
D	+ sodium pyruvate	J	$(NH_4)_2CO_3$ + sodium alpha-ketoglutarate
E	+ sodium oxalacetate	K	+ sodium pyruvate
F	+ glucose	L	sodium alpha ketoglutarate

1 alanine
2 glutamic acid
3 aspartic acid
4 mixture of amino acids, including methionine, phenylalanine, leucine, and isoleucine

to be dominant, *viz.*, the disappearance of alanine in Exp. 10 and 12 of Fig. 1 in which alpha-ketoglutaric acid was present.

Discussion. The importance of carbohydrates, in addition to their being a primary source of energy, in the metabolism of the rat tapeworm, *H. diminuta,* is indicated by the active participation of the degradation products of glucose in the synthesis of amino nitrogen from ammonia nitrogen. It is of interest to note, in relation to the essentially anaerobic environment of the worm, that the dominant pathway for this activity is through the amination of pyruvic acid to alanine. As has been shown above, this is quite different from the situation that is found in mammalian tissues.

The exact mechanism involved in the incorporation of ammonia nitrogen into the protein molecule is quite uncertain. Some investigators have suggested that this occurs in rat liver by a direct reductive amination of the keto acids(2). Others(7) have recently promoted a more complicated scheme involving the application of the Wood-Werkman condensation of pyruvic acid to oxalacetic acid, the reductive amination of a portion of this, the conversion of the remainder to other keto-acids via the tricarboxylic cycle, and a transamination with certain amino acids. The mechanisms of this synthesis in *H. diminuta* is now under study and it is hoped that information sufficient to describe the process in this organism will be obtained.

Read(8) demonstrated that enzymes catalyzing the oxidation of succinic acid and malic acid were present in homogenates of *H. diminuta.* The data obtained from the current study show that intermediary acids, such as alpha-ketoglutaric and oxalacetic, exist in worm tissues, and that enzymes responsible for their oxidation, and probably that of other acids in the tricarboxylic cycle, function effectively in the oxidation of pyruvic acid in the intact worm. It appears, however, that their activity does not approach the level which is found in such mammalian tissues as rat liver.

It may be observed in Fig. 1 that amino acids other than alanine, glutamic acid and aspartic acid are involved in the total gain in amino nitrogen following incubation with ammonium ions and the respective keto acids. At this time no explanation can be given as to how these other amino acids are related to those that are more directly produced by the incorporation of ammonia nitrogen.

Summary. 1. *H. diminuta,* in the presence of alpha-ketoglutaric acid, pyruvic acid, or oxalacetic acid and ammonium ions is capable of synthesizing amino nitrogen. It appears that this synthesis is so biased that the pyruvic acid-alanine system is favored. 2. Glucose, through its degradation to intermediary keto acids, is also capable of serving as a substrate for the synthesis of amino nitrogen from ammonium nitrogen. The yield, however, does not approach that of the more direct systems. 3. Evidence is given to show that the tricarboxylic cycle actively functions in the living intact worm.

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Effect of Parathyroid Extract Administration in Sheep.* (20858)

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Studies of parathyroid function and the effects of administration of parathyroid extracts have been confined primarily to 3 species of mammals, the rat, dog, and man. However, there is evidence to indicate that these glands function similarly in most mammals. Fleischmann(1) has reviewed the comparative physiology of the parathyroid glands. The availability of radioisotopes of phosphorus and calcium has facilitated investigation of parathyroid function in relation to blood levels, kidney function and particularly bone physiology. Minor differences have been noted in the bone uptake of Ca^{45} and P^{32} following parathyroidectomy or administration of parathyroid extract(2,3). Recently Talmage *et al.*(4) have shown that parathyroid extract administration to rats was able to remove previously deposited radiophosphorus to a greater extent than similarly deposited radiocalcium. Studies on the effect of parathyroid extract in sheep were therefore undertaken to determine species differences. In addition, the size of the animal permitted daily collection of blood and urine samples for analysis, and the large bones of the animal facilitated detailed autoradiographic observations of the isotope distribution pattern in the bone. It was hoped, therefore, that the locus of action of the parathyroid hormone could be more clearly elucidated.

Materials and methods. For this work, 19 ewes weighing approximately 75 lb were used. The radioactivity was injected either intravenously or intraperitoneally and at least one control was run concurrently with each group of experimental animals. The radioactivity was administered from one to 10 days prior to parathyroid extract‡ administration. The extract was injected subcutaneously at levels of 1000 i.u. and 3000 i.u. daily which corresponds to 30 and 90 i.u. per kg body weight. At the higher level the experiment was concluded at the end of 24 or 48 hours. At the lower levels animals were killed after 2, 5, and 7 days of treatment. Examination of the data showed little or no differences due to variation in dose or time of sacrifice and therefore all results are averaged for clarity of presentation. In some cases the animals were placed in metabolism cages for the collection of urine. Due to the comparatively large blood volume of the animals, frequent sampling was possible for serum calcium and phosphate determinations. At the conclusion of the experiment, the animals were sacrificed and the bones prepared for autoradiographs as described previously(5). Radioactivity measurements for P^{32} and Ca^{45} were made by standard procedures, serum calcium determinations by the method of Clark and Collip, and serum phosphate determinations by the method of Fiske and SubbaRow.

Results. The data from these experiments are tabulated in Tables I and II and the autoradiographs are pictured in Fig. 1. The data indicate that the sheep did not follow the pattern of other animals studied in its response to parathyroid extract and the various effects are summarized below.

Effect on serum phosphate and calcium. In all experiments the serum phosphate level was elevated by the injection of parathyroid

TABLE I. Inorganic Calcium and Phosphorus Serum Values for Controls and Sheep Treated with Parathyroid Extract.

	Ca, mg/100 ml	PO_4, mg/100 ml	No. of samples	No. of animals
	Mean and S.D.			
Control	11.7 ± .15	6.1 ± .16	24	7
Treated	11.6 ± .96	8.3 ± .80	23	12

* Published with permission of the Director of the University of Tennessee Agricultural Station.

† Oak Ridge Institute of Nuclear Studies Research Participant from Rice Institute, Houston, Texas.

‡ Supplied through the courtesy of Drs. D. C. Hines and R. M. Rice of Eli Lilly and Co., Indianapolis.

TABLE II. Optical Densities of Bone Autoradiograms from Controls and Sheep Treated with Parathyroid Extract.

	Optical density (mean and S.D.) Plate	Funnel	No. of samples Plate	Funnel	No. of animals	Level of sig. for mean diff.	% reduction* Plate	Funnel
P^{32}								
Control	1.250 ± .045	.848 ± .025	25	15	5			
Treated	1.064 ± .032	.745 ± .025	40	24	8	.01	62.0	34.5
Ca^{45}								
Control	.729 ± .053	.636 ± .048	10	10	2			
Treated	.754 ± .062	.631 ± .037	10	10	2	None	0	0

* A difference of .3 in optical density represents an increase or decrease of 100%.

extract (Table I). This is the reverse of what normally happens in rats, dogs, and man. The average serum phosphate for the control sheep used in these experiments was 6.1 mg % as compared with 8.3 mg % for the treated animals. There was no significant difference between the serum calcium levels of the control and experimental.

Effect on isotope excretion by the kidney. The renal excretion values for P^{32} and Ca^{45} in these experiments are incomplete. However, the results of these preliminary data are too important to exclude from this report. Based on 4 treated and 2 control animals given P^{32}, the total urinary excretion of the isotope by the treated sheep averaged only slightly over 2 times that of the control. This is a markedly small increase as compared to the 30-fold increase seen in the rat(4). Based on one experimental and one control animal, there was no significant difference in the renal excretion of Ca^{45}, however, previous studies in other species have never shown marked changes in calcium excretion following parathyroid extract administration.

Effect on removal of P^{32} and Ca^{45} from bone. As a result of parathyroid administration, a marked removal of P^{32} occurred from the primary spongiosa directly beneath the epiphyseal plate of the long bone and also from the endochondral bone of the funnel as shown in Fig. 1. It is of particular interest that radioactive phosphorus once deposited in the endochondral bone of the funnel is normally the last to be removed in the process of growth(6). Fig. 1 also demonstrates that there was no significant removal of Ca^{45} as a result of the same experimental treatment which caused such a marked P^{32} removal. The autoradiographic data for all the animals are summarized in Table II, which presents the mean densitometer readings for the autoradiograms. It is clear that the densitometric estimates support the visual observations as illustrated in Fig. 1.

Discussion. The most conspicuous difference in the effect of parathyroid extract on calcium and phosphate metabolism in the sheep as compared with other mammals was the failure of the extract to produce a large increase in phosphate excretion and to cause a decrease in phosphate blood level. The blood, urine and bone data obtained can be interpreted to give the following picture of parathyroid action: In the sheep, the parathyroid extract had a direct action on bone causing a release of phosphate, but there was little or no effect upon the kidney. This caused the blood phosphate to be increased, which may in turn have resulted in the slightly increased urinary excretion of phosphate which was observed. It is clear that in this case the removal from the bone was not a secondary effect caused by deficit of phosphate in the blood arising from the increased urinary excretion which does occur in rats and dogs.

There seems little question, both from the results with rats(4) and the data reported here, that more radiophosphate was removed from bone than was radiocalcium. In the light of present knowledge of bone formation and destruction it is difficult to conceive of a large effect on bone phosphate without a similar effect on bone calcium. It should first be noted that recently deposited Ca^{45} and P^{32} is probably located in the bone crystal in sites accessible for exchange or metabolic activity so that the removal of radioactivity

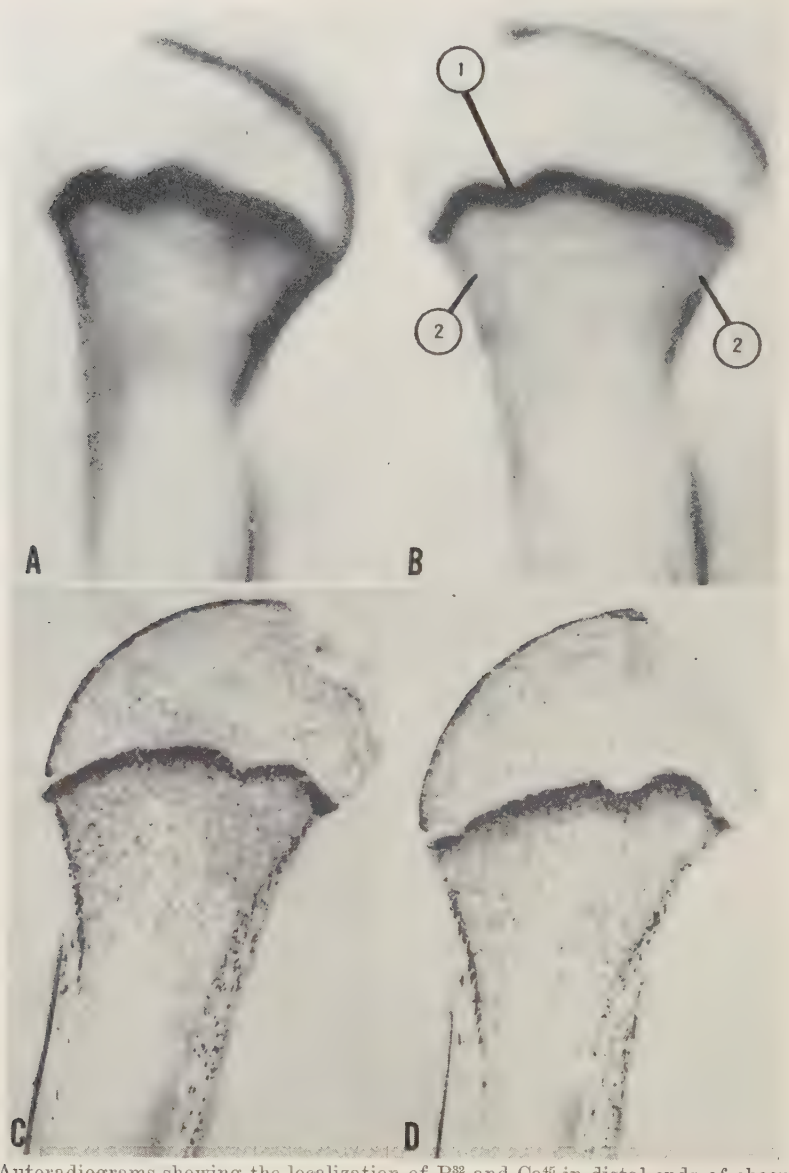

FIG. 1. Autoradiograms showing the localization of P^{32} and Ca^{45} in distal ends of sheep femurs. A. P^{32} in control sheep. B. P^{32} in sheep given 30 i.u./kg parathyroid extract; note removal from primary spongiosa (1) and from funnel (2). C. Ca^{45} in control sheep. D. Ca^{45} in sheep given 30 i.u./kg parathyroid extract; note the lack of removal from primary spongiosa and funnel.

does not represent stoichiometric removal of bone phosphorus and calcium. It may well be that the 2 elements at least in the active sites, are more independent of each other than has been generally realized and that parathyroid action is primarily concerned with phosphate ion and only indirectly with calcium ions.

Another possible explanation is that the parathyroid action causes the bone calcium and phosphorus to be placed in a state where ions of both elements are readily exchangeable with other body stores. Since there is a large pool of phosphate and very little calcium outside of bone, such a mixing of bone elements with the body stores would cause a large de-

crease in P^{32} and a very small decrease in Ca^{45} specific activities.

Summary. The effect of parathyroid extract administration upon the removal of radiocalcium and radiophosphorus from the long bones of sheep and its subsequent excretion in the urine was studied. Over a 5-day period of treatment, radiophosphorus but not radiocalcium was removed from the bone. The parathyroid extract produced only a slight rise in the urinary excretion which may be accounted for by the elevated serum phosphorus. Daily doses of 1000 i.u. of parathyroid extract over a 7-day period resulted in elevation of the serum phosphorus levels but not of serum calcium. It is concluded that in the sheep, parathyroid extract acted directly upon the bone and that any effect upon the kidney was minor in the dosage range and time periods employed; also, it appeared that phosphorus metabolism was much more influenced than was calcium metabolism.

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Prolonged Administration of Chloramphenicol in Monkeys. (20859)

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The relationship of chloramphenicol to blood dyscrasias in certain susceptible persons has been reviewed recently(1). To date no standard method is available to predict by experimental methods the potential hemocytotoxic effects in humans of new drugs. In earlier studies from this laboratory, the monkey has proved to be a valuable animal for hematologic study(2-5). It is the purpose of this paper to present the results of prolonged chloromycetin administration to monkeys.

Methods and materials. A total of 15 young adult *Macaca mulatta* were used in these preliminary studies. One group of 6 animals was administered 750 mg of chloromycetin daily except Sundays for 15 months from July, 1952 through October, 1953. The contents of 3 250 mg capsules were suspended in water, drawn up into a 50 ml syringe and introduced by stomach tube. Any residual material was rinsed through with water. Since these animals weighed between 5 to 6 lb at the beginning, the dosage approximated 250-350 mg/kilo. Two monkeys received a similar daily dose on alternate weeks for 15 months. Two had daily doses of 5 g and another 2 received 470 mg chloromycetin palmitate twice daily. Another pair of monkeys were started on 750 mg daily after receiving 200 r of total body irradiation. Three monkeys, after 2 months on vit. B-free synthetic diet, were given 750 mg chloromycetin daily along with a normal diet. Five to 7 complete blood counts were performed over a 2-week period before therapy, for base-line purposes. In addition, a pre-treatment bone marrow aspiration was also done. Serial blood counts (total 90 to 100) were then done daily during the first month and then bi-weekly thereafter. Bone marrow aspirations (total 6 to 7) were performed at approximately 2 to 3 month intervals. Chemical(6) and microbiologic(7) determinations of chloromycetin were done at weekly intervals to insure absorption of the drug. Weights were checked weekly.

Results. As can be seen in Fig. 1, 6 monkeys (A,B,C,D,E,F) given 750 mg daily re-

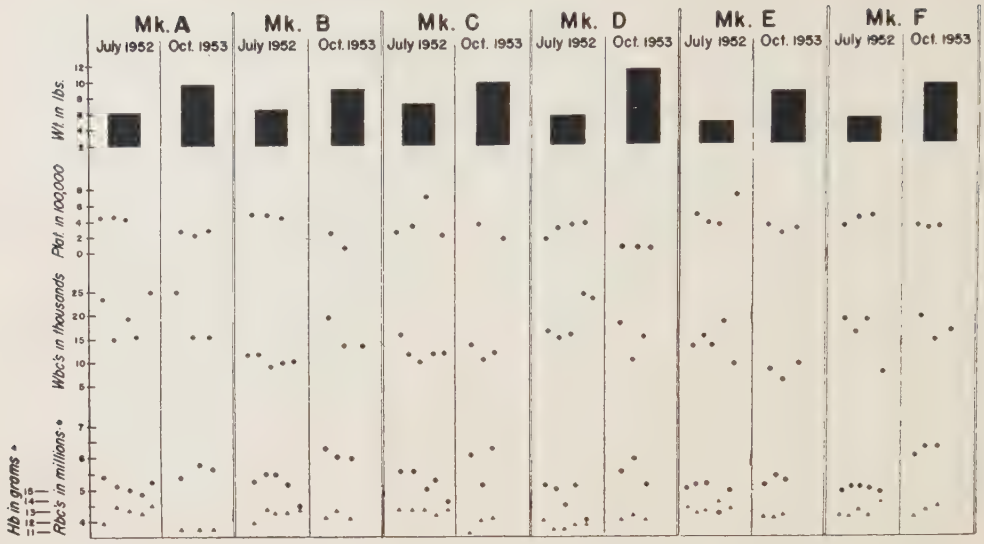

FIG. 1. Prolonged chloromycetin administration to monkeys (750 mg daily; total dosage, 300 g).

ceived a total of 300 g from July, 1952 through October, 1953. All monkeys gained weight on this regime. There were no significant alterations in any of the peripheral cellular elements as compared to the base-line values taken in July, 1952. No bone marrow depression or changes were noted in any of these animals. Similar results were obtained with set 2 (Monkeys G,H) which received 750 mg daily every other week with a total dosage in H of 150 g. Monkey G died after 14 months of drug administration with generalized tuberculosis. However, neither peripheral blood, nor bone marrow examinations were altered as compared to base-line studies. One (J) of the 2 monkeys in set 3 receiving 5 g daily died after 2 months. This animal had begun to refuse feed while on this program but ante and postmortem studies showed no hematologic abnormalities. Similarly, no abnormalities were noted in Monkey I which received a total of 1800 g over a 14-month period. Negative results were also observed in 3 monkeys (O,S,T) receiving 470 mg of chloromycetin palmitate twice daily for 7 months, for a total dosage of 188 g. Monkeys M and Z which were started on chloromycetin daily immediately after receiving 200 r of total body irradiation likewise were not adversely affected by the drug. Monkey M developed a temporary leukopenia between the 2nd and 3rd weeks after irradiation but the peripheral count slowly returned to normal as did that in Monkey Z (Fig. 2) which also had a temporary post-irradiation depression of red cells as well. Similar results were observed in 3 monkeys who after induction of a moderate nutritional cytopenia restored their hematologic equilibrium while on chloromycetin and normal diet. The blood of all of the monkeys in this study showed microbiologic and chemical evidence of absorption of the drug throughout.

Discussion. It is obvious that this series is small and the results preliminary in nature. Ideally, thousands of monkeys would be preferable, but that would not be practicable or economically feasible. Under the conditions of these studies, to date, we have not been able to produce any hemocytotoxic effects in monkeys. For the past 2 years similar intensive studies in patients receiving chloromycetin for disease processes in which the drug was specifically indicated have revealed no untoward hematologic effects. This is in agreement with other observations recently reported(8). There is still a need for a method whereby the potential bone-marrow depressing effects can be established for each new therapeutic agent. Other studies are in progress in this laboratory to attempt to learn why certain individuals have shown hemato-

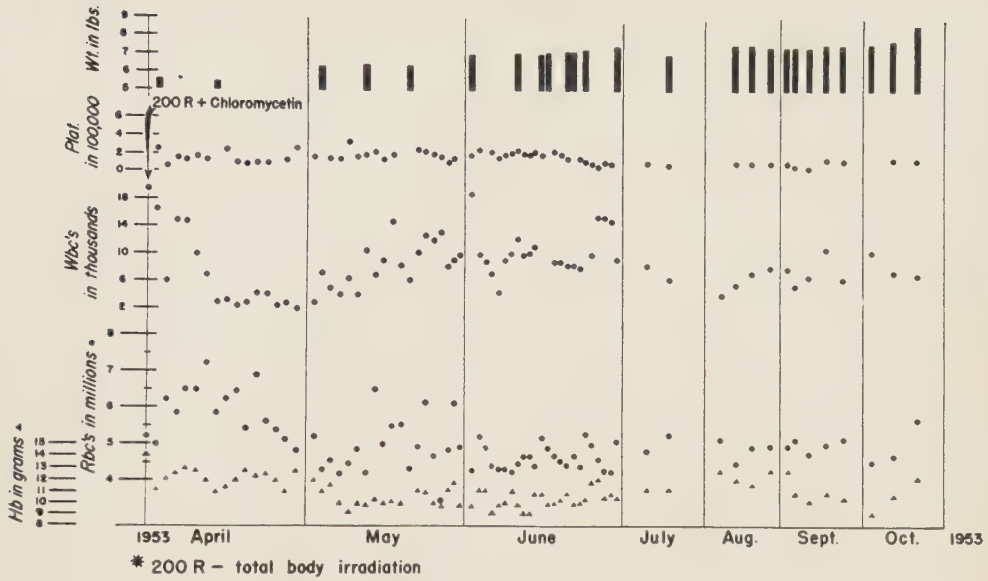

FIG. 2.
Chloromycetin in irradiated monkeys.* Mk. Z (dosage, 750 mg daily; total dosage, 126 g).

logic idiosyncrasies following chloromycetin therapy.

Conclusion. Prolonged administration of chloromycetin did not alter the peripheral or bone-marrow picture in normal, irradiated, or nutritionally cytopenic monkeys.

The authors gratefully acknowledge the technical assistance of Sue Blackburn.

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Development of Portal Fatty Liver in Rats on Corn Diets; Response to Lipotropic Agents.* (20860)

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During the course of a study designed to assess the effects on rats of diets related to those consumed by children in whom Kwashiorkor develops, we have noted the consistent occurrence of fatty liver. The lipid is predominantly in the portal area of the lobule in contrast to the centrally located fat usually observed in rats on diets low in choline and methionine(1-3). Clinicians familiar with Kwashiorkor in various parts of the world agree that the fat accumulates first in the portal areas of the liver lobule and disappears last from them following proper dietary treatment(4-7). It would appear obvious that the development of the analog of this human disease in experimental animals requires the production of initial lipid accumulation in the portal areas of the liver.

Methods and procedures. Black rats derived originally from a mixed stock† were used. Two main groups with different nutritional histories were studied. One group termed "first generation" was the offspring of stock breeding females maintained on Rockland "D-free" pellets. The majority was fed the stock pellets for one week after weaning (21 days) and then placed on the experimental diets. In one experiment the rats were placed on the experimental diet at weaning. The second group, termed "second generation" was obtained from mothers maintained on the experimental diets (supplemented in the majority of instances by weekly subcutaneous injections of 3 μg of vit. B_{12}) for at least one month before mating. These second generation rats were, in turn, placed on the experimental diets at weaning. They were used in an effort to obtain more severe deficiencies with more obvious biochemical and pathological changes than might occur in the first generation rats. Diet 19 had the following composition: whole "water-ground" bolted white corn meal 76,‡ casein§ 3, Crisco 15, 5% cod liver oil in corn oil 2, salts (Hubbell, Mendel, and Wakeman) 1, vitaminized glucose 3 which contributed (in mg): thiamine-HCl 0.5, riboflavin 1, niacin 5, pyridoxine-HCl 1, calcium pantothenate 2.5, folic acid 0.02, and biotin 0.01. Diet 23 had a similar composition except that the 15 parts of Crisco were replaced isocalorically by 33.9 parts of glucose. Diet 39 had the following composition: casein§ 9, glucose 67, salts 4, 5% cod liver oil in corn oil 2, Crisco 15, vitaminized cerelose (identical with that for diet 19) 3. Animals were killed by exsanguination following intraperitoneal pentobarbital (Nembutal) anesthesia. Lipids were measured in the entire liver except for small portions of the left and median lobes which were taken for histological study. Chemical analysis involved gravimetric determination of the fatty acids and unsaponifiable matter. The sections of liver for histological study were placed in 10% formalin within a few minutes after death; paraffin sections were stained with hematoxylin-eosin, and frozen sections were stained with Sudan IV for fat and with hematoxylin as a counterstain.

Results. The weight increase on diet 19 averaged 1 to 3 g per week for the first 2 months. Female rats, during the first month on these diets, grew as well as, or better than the males; thereafter most of the males slowly outgrew the females. Frequent graying of

* This study was generously supported by a grant from the National Vitamin Foundation, N. Y. City. Amino acids and most of B vitamins supplied by Merck and Co., folic acid by the Lederle Laboratories and cod liver oil by Mead Johnson.

† Obtained about 4 years ago from Dr. G. Emerson of the Merck Institute and since bred by one of us (MES).

‡ "Indian Head," Wilkins-Rogers Milling Co., Washington, D.C., purchased locally.

§ General Biochemicals, Inc., plain, untreated.

TABLE I. Microscopic Distribution of Lipid in Livers of Second Generation Rats on Corn Diet 19. (No. of rats and location of lipid in lobule.)

Diet	Sex	Total No.	Portal		Central		Diffuse†	Little or none
			Definite	Tendency*	Definite	Tendency*		
19	♂	23	13	1	1	0	8	0
	♀	17	8	2	1	1	4	1
19 + B_{12} (3 μg weekly)	♂	15	8	0	0	0	2	5
	♀	14	5	0	0	0	0	9
19 + 0.5% methionine	♂	9	7	0	0	0	0	2
Totals		78	41	3	2	1	14	17

* Tendency = fat occurred throughout lobule but was most prominent in region indicated.
† Diffuse = fat occurring throughout lobule without obvious localization.

hair was noted. Symmetrical alopecia of varying duration often occurred, and, when hair growth resumed, the new hair was gray or even white and of a fine soft texture; this new hair gradually became pigmented(8).

Gross and microscopic appearance of liver. Primary attention has been devoted to second generation rats on diet 19 in whom portal fat occurs quite consistently (Table I). Grossly, many of these livers had a "mottled" appearance with the lace-like yellow or light tan of the fatty liver surrounding tiny red or brown dots or streaks. This pattern was seen particularly consistently in the rats treated with 0.5% DL-methionine or vit. B_{12} and in which only a partial lipotropic response had been obtained (*vide infra*). Upon microscopic examination sudanophilic material was found predominantly in the portal areas of the lobules (Fig. 1). In general, the cells contained many small or medium sized fat droplets. In paraffin sections stained with hematoxylin and eosin the fat-containing cells had a "foamy" type of cytoplasm—a type of vacuolization different from that caused by glycogen. As the fat content became greater, large droplets of sudanophilic material were seen displacing the nucleus well to the side of the cell. When the lipid content of the liver rose above 10 to 15% as determined by chemical analysis, the fat was found to extend throughout the lobule. Administration of

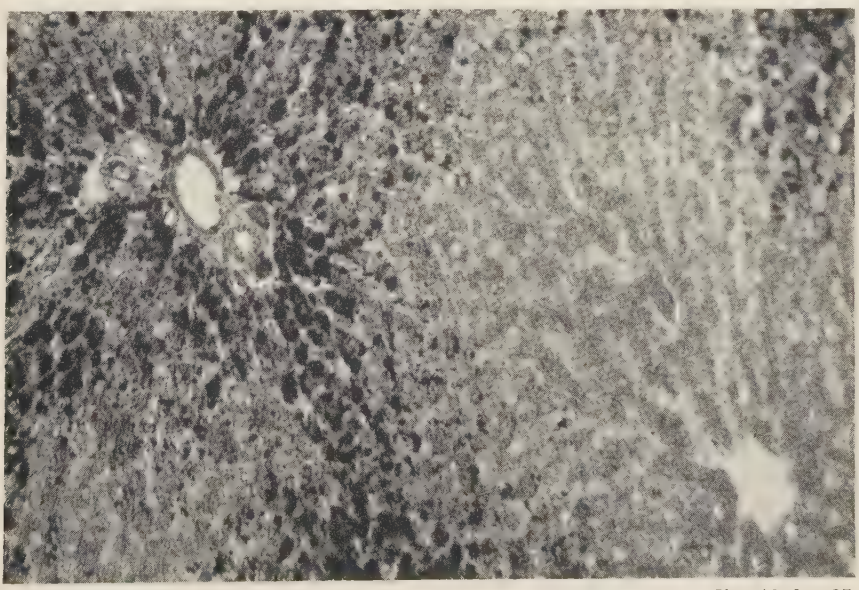

FIG. 1. Section of liver of a second generation male rat subsisting on corn diet 19 for 65 days. At upper left is a portal area, at lower right a central area. Sudanophilic material (black) is limited to portal portion of the lobule. Sudan IV and hematoxylin (× 300).

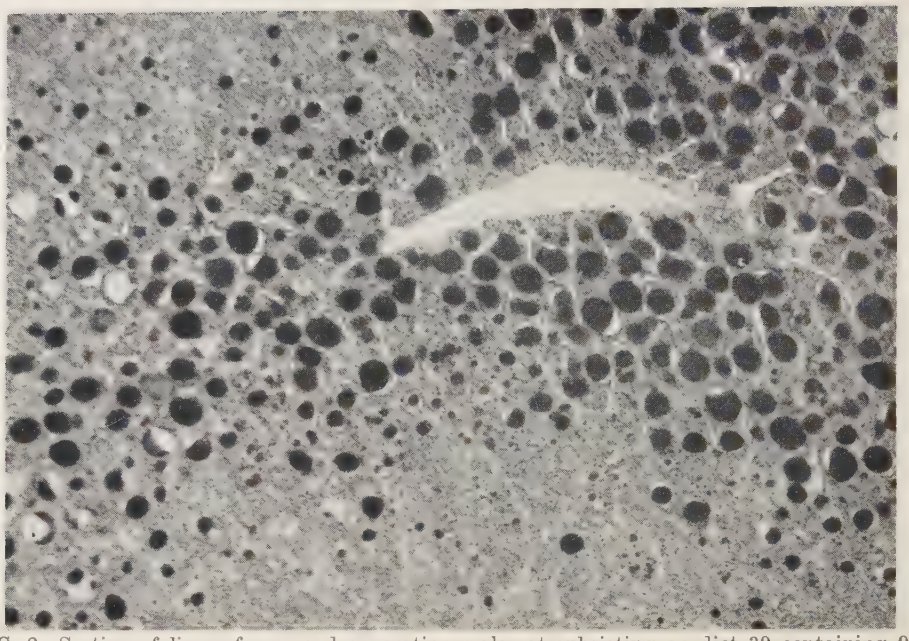

FIG. 2. Section of liver of a second generation male rat subsisting on diet 39 containing 9% casein. At upper right is a central vein. Large black sudanophilic globules cluster about this vein. Portal areas of this lobule were free of fat. Sudan IV, hematoxylin (× 300).

lipotropic substances which did not remove all of the fat resulted in a sharp portal localization of fat in these second generation rats.

It was possible that the portal accumulation of fat was a peculiarity of the strain of rat used in these experiments. To check this, rats of both sexes were placed on diet 39 in which the only source of protein was casein and in which choline was lacking. First and second generation‖ littermate rats were fed either diet 19 or a related diet containing 2% more salts and only 1.5% casein. These animals were carried for 11-60 days on the diets. DL-methionine (0.5%) or choline chloride (0.25%) were incorporated in the diets of some of these rats for 3 to 5 days before sacrifice in an attempt to localize the liver lipid more sharply. The livers of 31 rats on diet 39 have been examined. Twenty-four had lipid either localized in the central areas or with the largest lipid droplets occurring centrally (Fig. 2). The remaining 7 rats had fat distributed throughout the lobule with no discernible localization. The rats on the high corn diets had the fat in the periphery in every instance except where the lobule was so loaded with fat that no localization was discernible. Thus it appears that initial or preponderant accumulation of lipid in the portal areas is related to the presence of corn in the diet.

Lipid concentrations in the liver and the effects of lipotropic agents. Chemical analyses of the livers revealed amounts of lipid which corresponded well with the microscopic picture. The percentages found varied within the rather wide ranges which many investigators have noted in experimental fatty livers on a variety of diets.

Diets consumed in areas where Kwashiorkor exists tend to be low in fats. Consequently, it was thought desirable to study the effect on liver lipid accumulation of low versus moderate amounts of fat in corn diets. Such a study using first generation rats on the high corn diets (diet 19 with 17% by weight of fat versus diet 23 with 1.7% fat) revealed no appreciable difference, either in the fat content of the liver, or in rate of growth (Table II, exp. 1.) A further experiment extending

‖ Second generation rats placed on diet 39 were rats from mothers on the corn diet.

TABLE II. Comparison of Liver Lipids and Growth in Rats of the First Generation on Diet 19 and Effectiveness of Lipotropic Agents.*

Exp.†	Supplement	Days on Diet	Days on Supplement	% liver lipids Range	% liver lipids Avg‡	Avg wt gain (g)
1 ♂	—	92–115	—	9.6–22.9	13.8 (7)	149 (7)
	§	"	—	10.2–18.2	12.4 (5)	151 (5)
2 ♂	—	92–115	—	9.6–24.3	15.3 (12)	—
	Meth.‖	"	27– 37	3.5– 7.7	5.4 (6)	19: 39 (9)††
						19 + meth.: 39 (9)††
	B_{12}¶	"	92–115	2.7– 4.1	3.4 (8)	19: 148 (8)‡‡
						19 + B_{12}: 150 (8)‡‡
	Casein**	"	92–115	3.2– 3.6	3.5 (4)	19: 135 (6)‡‡
						19 + casein: 355 (6)‡‡
3 ♂ + ♀	—	65	—	—	—	117 (6)
	Meth.‖	65	65	—	—	104 (6)
4 ♂	—	255	—	20.9–24.1	22.5 (7)	—
	Meth.‖	"	31	11.3–13.9	13.1 (4)	—

* Within each experiment litter mates distributed among diet groups to compensate for litter differences in liver fat accumulation and wt gain.

† Exp. 1 and 2 started at 28 days of age; Exp. 3 at 21 days; Exp. 4 at 45 days.

‡ ( )—No. of rats.

§ 15 parts of Crisco were replaced isocalorically by 33.9 parts of glucose (diet 23).

‖ 0.5% DL-methionine in diet.

¶ 3 μg vit. B_{12} inj. intraper. once weekly.

** 15 g of GBI casein added to each 100 g of diet 19.

†† Wt gain for the last 27-37 days of exp. Rats with methionine compared with littermates on diet 19 only.

‡‡ Wt gain over period of 90 days. Rats with supplement are compared to littermates on diet 19 only.

the time on these diets to 191 days confirmed this finding.

The effect of lipotropic substances in the prevention and cure of fatty liver was considered of interest in view of the occurrence of portal fat. The findings with first generation rats are given in Table II. It is seen from the data (exp. 2) that vit. B_{12} and casein were very effective in preventing fat from accumulating in the liver. DL-methionine (0.5%) added 27-37 days before the end of the experiment reduced the amount of fat; however, in 5 of the 6 rats the concentration was still above normal. When 0.5% methionine was added to the diet of rats during the last month of an 8½-month period on diet 19 (exp. 4), an appreciable reduction in lipid occurred, but considerable fat still remained. Preliminary evidence suggests that choline chloride (0.25%) was as active as vit. B_{12}.

It is apparent that diet 19 was deficient in choline and substances that yield or allow formation of labile methyl groups for choline synthesis. Methionine, and vit. B_{12} were not limiting factors for growth in this diet (Table II, exp. 2 and 3) whereas casein caused a marked increase of growth and prevented excessive accumulation of fat in the liver (Table II, exp. 2).

Methionine, choline and vit. B_{12} were also effective in reducing liver fat in second generation rats. However, here again, one-half percent methionine was rarely completely effective in preventing accumulation of fat (Table III). This level of methionine did not improve growth. Increasing the level to 1.0% did not appreciably improve the lipotropic action and depressed growth in some of the animals. In the small number of animals thus far tested, choline effectively prevented fat accumulation.

Vit. B_{12} injected into second generation rats was erratic in its lipotropic activity. In some rats it was completely effective in preventing fatty livers; in others considerable fat accumulated. In all instances, however, administration of this vitamin resulted in lower lipid levels than in the untreated rats. An example

TABLE III. Effect of Methionine and Choline on Liver Fat in Second Generation Rats.*

Supplement	Exp. 1 None	Exp. 1 .5% methionine	Exp. 1 .25% choline Cl	Exp. 2 .5% methionine	Exp. 2 1.0% methionine
No. animals	6†	11‡	5§	7♂	9♂
Littermate wt gain (g)	104	102	—	86	69
Avg % lipid (wet wt)	15.3	6.2	3.4	7.2	5.5
Range	8.4–24.4	4.0–9.1	3.2–3.8	3.0–11.2	3.7–8.4

* Diet 19 begun at 21 days and continued for 65 days. † 4♂, 2♀. ‡ 8♂, 3♀. § 3♂, 2♀.

of the results obtained is given in Table IV for littermates weaned by mothers subsisting on diet 19. Similar results have been obtained with young from females on diet 19 supplemented with vit. B_{12} with or without additional salt mixture. In this generation, as in the first, B_{12} did not improve growth despite its lipotropic action and despite the fact that these second generation rats came from mothers who had not received the vitamin for several months or longer. In 11 out of 12 female littermate pairs, the animal receiving B_{12} grew less than its sister not receiving this vitamin. In male littermates there did not appear to be any consistent trend.

Discussion. While methionine was not completely effective in preventing some accumulation of fat, it had significant lipotropic action in our diet 19. Similarly, Dean(9) noted that unspecified amounts of methionine prevented fatty liver in weanling rats fed *matoke.*¶ Our "mottled" livers resemble some of the fatty livers described by Gillman *et al.* (10) in rats on a diet of cooked corn meal and sour milk. Some of their rats also had portal fat. Corn cannot be the unique etiological factor in the development of the portal fatty liver of Kwashiorkor because the liver changes occur even under conditions where corn is not an important dietary component. However, corn appears to have a characteristic in common with some other plant materials that predisposes to the syndrome under certain nutritional conditions. Our experiments emphasize the fact that there is some factor (or factors) either present or absent in corn which results in portal rather than central fat. It is hoped that the ability to develop the portal type of fatty liver in the rat may help shed light on some of the nutritional factors operating in Kwashiorkor.

TABLE IV. Effect of Vit. B_{12} on Hepatic Lipid and on Body Weight Gain in Second Generation Rats from Mothers on Diet 19. (Young started at 21 days, killed after 65 days on diet.)

Litter No.	Sex	No supplement % lipid	No supplement Wt increase (g)	Vit. B_{12}* % lipid	Vit. B_{12}* Wt increase (g)
356	♀	14.0	78	9.2	68
	♀	19.7	89	—	—
399	♀	11.1	94	3.4	81
	♂	5.6	125	5.0	136
403	♀	16.8	103	11.6	99
416	♀	21.2	120	13.0	121
	♂	10.1	170	6.4	146
417	♀	5.6	128	3.0	79
	♀	8.3	104	3.4	96
	♀	—	—	4.7	94
	♀	—	—	11.3	79

* 3 μg vit. B_{12} inj. intraper. once weekly.

Summary. Rats subsisting on a diet containing 76% corn and 3% or less casein, developed a fatty liver characterized by initial and preponderant accumulation of lipid in the portal areas. Rats of the same strain on a diet in which the only protein was casein, developed a centrolobular type of fatty liver. Methionine, choline, and vit. B_{12} were able to decrease the liver lipid. However, methionine was rarely capable of preventing the deposition of some excess fat while, under certain conditions, the lipotropic action of vit. B_{12} was unpredictable.

¶ Cooked banana, an important constituent of the diets of children in Uganda, among whom Kwasiorkor occurs.

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The Adequate Stimulus for Shivering.* (20861)

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In man, acute exposure to cold is a stimulus for a heat conservation mechanism (vasoconstriction) and a heat production mechanism (increased metabolism). The response of the vasoconstrictor mechanism to decreasing environmental temperature has been repeatedly demonstrated by both skin temperature(1) and blood flow measurements(2). Although the adequate stimulus can be either local or general body cooling, it is usually local skin cooling, vasoconstriction occurring before deep body temperature changes. Previous exposure to cold, however, modifies this response(3-5). The mechanism inducing increased metabolism upon acute exposure has been the subject of controversy for almost a century. The argument is still much as Cannon *et al.*(6) stated it: whether there is a true chemical regulation as Rubner(7) defined it in addition to the metabolic response to increased muscular activity in the form of involuntary shivering. The work from Cannon's laboratory gave evidence that a cold stimulus causes increased metabolism when there is no muscular activity. This was ascribed to the release of adrenalin. Subsequent work has failed to confirm these results, whether the negative heat load was from the external environment(1) or from internal cooling(8). Cannon had hinted that acute rapid application of the negative heat load gives rise to early shivering which masks the chemical regulation, but the data of Hardy and DuBois(1) and Pinson and Adolph(8) do not bear out this suggestion. A steady state was approached in both series of experiments, in the former with a lowered skin temperature and a maintained rectal temperature, and in the latter with a lowered rectal temperature and less change in skin temperature. However, during long-term exposure of animals to cold, shivering seems to decrease while the increase in metabolism is maintained (9). Initiation of shivering has been attributed to a fall in internal body temperature (rectal)(10,11) or in skin temperature(12). Hensel's(13) recent account of the thermal sense organs gives a basis for predicting that shivering is the result of the rate (depending on steepness of thermal gradient) at which cold sensitive end organs are discharged, the number (depending on area and density of end organs) being stimulated and the thermal state of the temperature regulating center.

The following experiments were conducted to determine if a true chemical regulation could be demonstrated and if shivering is controlled according to Hensel's description.

* Supported by contract between the University of Washington and the Alaskan Air Command, Arctic Aeromedical Laboratory, Ladd Air Force Base, Fairbanks, Alaska.

Methods. Skin temperature, heat loss, oxygen consumption and blood flow were recorded in four normal subjects. All experiments were conducted 2 to 3 hours after breakfast during June, July and August. The subjects lay supine on a cot with a plastic screen in place of canvas, in a room with controlled temperature and a fixed air flow. After control records were made at 24-25°C, the room was cooled at a rate of 0.6°C per minute. In some experiments, the subject drank a liter of ice water just before the room temperature was reduced. Skin temperatures were measured with copper-constantan thermocouples of 30 gauge B & S wire and recorded on a Brown potentiometer with a –0.5 mV to +2.5 mV range. Thermocouples were located at the points recommended by Hardy and DuBois(1), those from corresponding parts of the body surface being connected in parallel to give an average reading. To record heat loss single Hatfield tellurium discs were used on the hand and foot, and for the chest and on the thigh and legs four elements connected in series were used. Oxygen consumption was determined by measuring minute volume with a linear flowmeter and with a Pauling oxygen analyzer (range 110-160 mm Hg). Blood flow in the first two phalanges of the right middle finger was determined by an air plethysmograph with a condenser pickup. The occlusion cuff, proximal to the finger cot, was inflated to 46-50 mm Hg.

Results. The data obtained are shown in Table I. The sequence of events was roughly skin cooling, reduced blood flow, shivering, and increased oxygen uptake. The first 2 were almost simultaneous and subjects often first reported shivering at the same time as their oxygen uptake increased. The position of reduced deep body temperature in this sequence is not definite. Decreased rectal temperatures sometimes preceded, sometimes followed the onset of shivering. In any event, lowered deep temperature does increase the tendency to shiver. The average time to onset of shivering was six minutes less when shorts-clad subjects drank ice water. At no time did shivering reach a level sufficient to maintain total body temperature.

By covering a region of the body, the rate

TABLE I. Average Data from 4 Subjects Tested for Shivering Response.

Approximate area exposed	Time to shiver, min.	Change in avg temp. (°C) of		Heat debt, kilo-calories	Change in rectal temp., °C	Heat loss during cooling‡				Max met.		Avg time to inc. in O_2 uptake, min.
		Skin	Thorax	Thigh				Heat loss during control				Control met.	
								Thorax	Leg	Hand	Foot		
Shorts, 100%		(31.8)*	(33.0)	(31.8)				(45)	(56)	(47)	(35)		
		2.9	1.8	3.1	0			2.5	2.7	3.4	2.7	1.7	
	14	(1.2–4.4)	(1.0–2.2)	(2.0–4.4)	44								19
	(12–20)†												
Thorax & arms		(33.5)	(34.6)	(32.3)				(20)	(40)	(60)	(60)		
covered, 51%		4.5	1.0	7.0	0.2			3.0	5.0	2.0	2.3	1.8	
	31	(2.6–6.2)	(.6–1.7)	(3.0–10.0)	59								31
	(17–47)												
Legs & feet		(33.0)	(33.0)	(33.3)				(50)	(20)	(70)	(22)		
covered, 41%		2.4	3.2	.8	0			5.8	3.0	2.0	3.1	1.9	
	18	(1.4–3.5)	(1.7–4.0)	(.2–1.0)	31								25
	(8–28)												
Ice water,		(32.3)	(33.1)	(31.7)				(57)	(55)	(51)	(50)		
shorts, 100%		1.5	1.1	1.3	0.9			1.9	2.4	1.6	2.0	-	
	8	(.7–2.6)	(.7–1.7)	(.8–2.0)	41								-
	(2.5–12.5)												

* Skin temp. during control period in parentheses. † Range in values. ‡ Figures in parentheses give control period heat loss in $Cal/M^{-2}/hr.$

of cooling there was reduced below that given by Hardy and Oppel(14) as the adequate stimulus for cold sensation. As can be seen from the Table, the time to onset of shivering was related to the regions of the body exposed. Covering the thorax delayed the onset of shivering longer than covering the legs. The relative rates were analogous to the relative distribution of cold spots(15). The estimated ratio of the number of cold spots in the trunk versus those in the legs is 6 to 4, as is the reciprocal of the time to shivering in the present experiments.

Discussion. Since the increased oxygen uptake noted was always subsequent to subjective reports of shivering, our data do not indicate the existence of a true chemical control of metabolism under the present conditions of cooling. The data are consistent with the hypothesis based on Hensel's description of thermoregulation, but pose a perplexing question in the long durations of stimulation necessary to occasion shivering. A possible explanation is the fact that cold-sensitive end organs sense absolute temperature as well as respond to rate of change. Alternative explanations are that the temperature regulating center can summate over long periods, or that changes in temperature of the brain occurred not demonstrated by rectal temperatures.

Summary. On the basis of Hensel's account of thermal sense organs, it may be postulated that adequate stimulus for shivering is a combination of the rate at which cold sensitive end-organs are discharged, the number stimulated, and the thermal state of the center. In some experiments, subjects drank cold water to lower deep body temperature before the start of room cooling. Skin and deep body temperatures, oxygen uptake, and finger blood flow were measured. In 4 subjects, the sequence of events with room cooling at 0.6°C/minute from 25°C was: skin cooling, reduced blood flow, shivering, and increased oxygen uptake. Lowered deep body temperature does increase the tendency to shiver. At no time did shivering reach a level to maintain total body temperature. Onset of shivering was related to regions of body exposed. Covering the thorax delayed its onset longer than covering the legs. The geometric areas exposed were comparable, a difference existing in the number of cold spots/cm^2.

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Influence of Total-Body X-Irradiation upon Tissue Mast Cell Number. (20862)

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We have previously reported(1) that total-body X-irradiation elicits marked changes (vacuolation, granule clumping and cell disruption) in the tissue mast cells of the cheek pouch of the hamster. In addition we noted our impression of a decrease in the total number of mast cells during the time when large numbers of atypical cells were present. Similar mast cell changes have been claimed in aged tissue cultures(2), edematous tissue and urticaria pigmentosa lesions(3), and treatment with toluidine blue(4), ACTH(5), and cortisone(5,6). More recent reports strongly indicate that mast cell changes must be evaluated quantitatively (*i.e.* by cell counts) in order to be meaningful, since no changes in mast cell number were found after ACTH, cortisone, or cold(7) or after cortisone or thyroxine(8). Moreover, it is claimed(7,8) that atypical mast cells occur as frequently in control as in experimental animals. The positive conclusions of the previous reports (1-6) were in no instance based upon mast cell counts. The present paper is concerned with results obtained from counting both normal and atypical mast cells in tissues of animals exposed to X-irradiation.

Methods. Young adult, male Syrian hamsters were used. Tissues (cheek pouch) were taken immediately upon sacrifice at various intervals after single total-body X-irradiations and were fixed in alcohol and stained in toluidine blue as previously described(1). The radiation factors were 250 kv, 15 ma, 0.5 mm Cu, and 3.0 mm Bakelite filters, 26.7 cm target distance, 1.5 mm Cu half-value layer and 215-225 r per minute. Tissues from non-irradiated hamsters kept under the same conditions as the experimental animals were simultaneously studied. Mast cell counts were carried out on the fixed tissues, separate account being taken of normal cells and atypical cells (cells with vacuoles and clumped granules(1)). Tissues were taken from the same region of the cheek pouch in all instances. On a single slide from each animal thirty contiguous fields, 0.0676 mm^2 each, were covered using a magnification factor of 264.

Results. The results are presented in Table I. X-irradiation causes a marked increase in the number of atypical mast cells and this is accompanied by a considerable decrease in the total number of mast cells. We find similar increases in the number of abnormal cells in the skin of rats given total-body exposures of 600 r(9). Thus our prior claim(1) that such changes follow exposure to X-rays is substantiated. That the occurrence of atypical cells is not a consequence of fixation, staining or manipulation is assured by the fact that we encounter such cells in large numbers in the intact mesentery of living anaesthetized animals previously exposed to X-rays(9). Abnormal mast cells of this kind are rarely seen in living preparations of non-irradiated controls.

The present data also support our previous claims(1) as to the times of disappearance of damaged cells and of repopulation of the pouch with mast cells. The counts indicate, however, that repopulation is slower than previously supposed and is not complete even at the end of 33 days.

It is of interest to note that nitrogen mustard is reported(7) to produce similar atypical mast cells in subcutaneous tissues in the rat, thus indicating another radiomimetic

TABLE I. Influence of X-Irradiation (600 r) upon Number of Mast Cells in the Hamster Cheek Pouch.

Day after exposure	Irradiated		Control	
	Total No. cells*	% abnormal cells*	Total No. cells	% abnormal cells
3	1177†	2.8†	1096	1.6
10	759	45.4		
17	363	5.5	1395	1.0
26	530	6.2		
33	776	3.9	1240	1.5

* Per 2.028 mm^2 of tissue.
† Avg of 3 animals.

action of these compounds.

Summary. 1. Mast cell counts were made on the cheek pouches of hamsters subjected to total-body X-irradiation. 2. Following irradiation there is a marked increase in the number of atypical mast cells accompanied by a reduction in the total number of mast cells. 3. Repopulation of the cheek pouch with mast cells is relatively slow, being incomplete even at the end of 33 days.

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Vitamin B_{12} and Transmethylation in the Baby Pig.* (20863)

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It has been stated by Schaefer *et al.*(1,2) that vit. B_{12} is required by the chick and the rat for the synthesis of choline from methionine. Stekol *et al.*(3) have stated, however, that "vit. B_{12} deficiency had no effect on the extent of transmethylation to choline or creatine". Johnson *et al.*(4,5) have shown that baby pigs raised on a synthetic milk diet containing adequate vit. B_{12} and 0.8% methionine require 0.1% choline in the diet and that this choline requirement can be eliminated by increasing the methionine level to 1.6%(6) as is true for the rat(7). Since a severe vit. B_{12} deficiency can readily be produced in the baby pig(8,9), the effect of this deficiency on choline synthesis by transmethylation from methionine was studied in this species.

In the previous work in which a high

TABLE I.

Basal diet	
Alpha-protein	29.4
DL-methionine	1.3
Cerelose	30.5
Lard	30.5
Minerals	8.3

The above materials were made into a synthetic milk containing 13% solids according to a modification of the W. M. Clark method(10).

Vitamins added/liter synthetic milk	mg
Thiamine	.65
Riboflavin	1.30
Pyridoxine HCl	1.30
Nicotinic acid	2.60
Ca pantothenate	7.80
Folic acid	.052
Biotin	.010
2 methyl-1,4-napthoquinone	.26
Vit. A	2000 I.U.
Vit. D	200 I.U.

* This work was supported in part by grants from the National Vitamin Foundation, New York, and from the Moorman Manufacturing Co., Quincy, Ill.

The vit. B_{12}, choline, thiamine, riboflavin, pyridoxine HCl, nicotinic acid, and calcium pantothenate used were generously supplied by Merck and Co, Rahway, N. J., through the courtesy of Dr. Harold H. Draper; the folic acid by Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y., through the courtesy of Dr. T. H. Jukes; the "water-soluble" vit. A and D, as A CON and D CON, by Endo Products Co., Richmond Hill, N. Y. through the courtesy of Dr. S. M. Gordon; the DL-methionine by the Dow Chemical Co., Midland, Mich., through the courtesy of Dr. J. E. Johnson; and the lard, as a stabilized 60% lard in water emulsion, by Armour and Co., Chicago through the courtesy of Mr. Bryon M. Shinn.

TABLE II. Growth, Liver B_{12}, Liver Choline, and Urinary Choline of Pigs.

Group	Avg initial wt, kg	Avg 4 wk wt, kg	Avg liver choline $-B_{12}$ mg/g fresh	Avg liver choline $+B_{12}$* mg/g fresh	Avg liver B_{12} $-B_{12}$ mμg/g fresh	Avg liver B_{12} $+B_{12}$* mμg/g fresh	Avg urinary choline excr. $-B_{12}$ mg/24 hr	Avg urinary choline excr. $+B_{12}$* mg/24 hr
1 Positive control ($+B_{12}$)	1.93	10.19	—	2.03	—	290	—	—
2 Basal	1.91	5.41	2.63	2.37	18.6	39.7	2.84	2.31
3 Basal + dimethylaminoethanol	1.60	4.71	2.61	2.38	18.1	48.6	25.84	17.85

* Four pigs, 2 in Group 2 and 2 in Group 3, given one dose of 50 μg of vit. B_{12} by injection at 4 wk. The $+B_{12}$ refers to these pigs in Groups 2 and 3.

(1.6%) methionine diet replaced choline(6), casein served as protein source and all the animals received vit. B_{12}. In the present experiments, alpha-protein supplemented with methionine, again to a level of 1.6%, was used as protein source to produce a B_{12} deficiency. It was found that the high level of methionine served as an adequate choline source even in animals moribund with B_{12} deficiency.

Experimental. Two groups (2 and 3) of 5 pigs each were fed *ad libitum* a high methionine alpha-protein synthetic milk diet, the composition of which is given in Table I. The choline content of the diet was determined to be less than .01% by the method of Horowitz and Beadle(11) using *Neurospora crassa* cholineless. The pigs in Group 2 received only the basal diet; and those in Group 3 received 0.1% dimethylaminoethanol to supply a methyl acceptor in the event that B_{12} deficiency blocked its synthesis. Group 1 was a positive control group; these 5 pigs receiving the alpha-protein basal with 0.5% methionine added (instead of 1.3%) as well as 0.1% choline and vit. B_{12} at the rate of 0.8 μg/kg body wt/day. After 4 weeks on the diet, 4 of the deficient pigs were injected with a single dose of 50 μg B_{12}/kg B.W. All the animals were killed when it became evident they were near death due to the deficiency. Liver and kidney samples were taken for histological examination and liver samples for choline and vit. B_{12} assay. Twenty-four-hour urines were collected prior to and after injecting the 4 deficient pigs and assayed for choline.

Results. The growth data given in Table II illustrate the effect of the B_{12} deficiency. The 4 pigs, which were injected with 50 μg B_{12}/kg B.W. after 4 weeks on the deficient diet, gave a characteristic reticulocyte response which reached a peak at 8 to 10 days as shown in Table III.

Choline assays of the urines (Table II) gave significantly higher results for the group receiving 0.1% dimethylaminoethanol due to its excretion and failure of the *Neurospora* assay to differentiate between it and choline. Injection of B_{12} failed to produce any difference in choline excretion.

The deficient pigs were killed during the fifth week of the experiment when they refused food, vomited repeatedly, and became very weak and unsteady. It has been repeatedly noted that B_{12} deficient pigs die soon after these symptoms are observed.

Histological examination of liver and kidney gave no evidence of fatty infiltration or renal damage indicative of a choline deficiency. Choline assay of the liver samples as indicated in Table II further shows that a choline deficiency did not exist in either Groups 2 or 3 as the values are essentially equal to those of Group 1 which received choline.

Marked differences were found in assays of the livers for B_{12}(12) (Table II). Controls receiving 0.8 μg B_{12}/kg B.W./day by weekly injection contained approximately 10 times more B_{12} than did the deficient animals. Slight increases were found in the 4 treated pigs.

Growth data and liver assays for B_{12} indicate that a definite B_{12} deficiency was produced while histological examination of livers and kidneys gave no evidence of a choline deficiency. It thus appears that in the pig, B_{12} is not involved in the synthesis of choline from methionine.

TABLE III. Reticulocyte Response to B_{12}. (Reticulocytes/100 red cells.)

Days after B_{12}	Basal		Basal + dimethylaminoethanol	
0	1.5	2.8	2.0	1.9
4	2.9	5.5	4.8	4.4
6	6.4	8.5	5.8	5.0
8	9.9	10.5	7.8	10.0
10	8.4	12.1	5.2	8.7
13	6.1	6.5	3.3	3.8

Summary. The effect of a B_{12} deficiency on transmethylation from methionine to choline has been studied. The deficiency did not influence the level of choline in the liver nor the excretion of urinary choline. Histological examination of livers and kidneys did not reveal the fatty infiltration or renal damage characteristic of a choline deficiency. From this study it appears that vit. B_{12} is not involved in this direct transmethylation.

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Histochemical Changes in Succinic Dehydrogenase Activity in Rat Kidney Following Administration of Mercurial Diuretics.* (20864)

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It is a well known fact that the sulfhydryl-containing enzymes are inhibited by compounds of heavy metals such as mercury(1). Consequently, succinic dehydrogenase activity has been shown to be inhibited by mercurial diuretic agents *in vitro* in the heart (2) and *in vivo* in the kidney(3). Such enzymatic inhibition is concomitant with the diuretic action of mercurials and may be related to the depression of tubular reabsorption, since both enzymatic inhibition and diuresis can be prevented by the administration of 2,3 dimercaptopropanol (BAL)(4).

It is generally assumed that mercurial diuretics act on some specific portion of the kidney tubule, although there is no satisfactory physiological evidence on this point (5). Recently, Mustakallio and Telkkä(6) using a histochemical method, reported that the "distal segment" of the tubule is inhibited by mercuhydrin. Since pathological and other evidence is not in agreement with their conclusions, we restudied the problem by an improved histochemical method and, in addition, investigated the activity of BAL, cysteine, and glutathione in suppressing the mercurial inhibition.

Methods. Nearly all of our work has been done on young female rats (175-200 g) of the Holtzman strain although kidneys of a few adult males and immature animals of this strain also have been studied. The animals were killed by decapitation, a kidney removed immediately, and sectioned at 50 μ on the freezing microtome. Sections were placed in physiological saline solution and within a period of 30 minutes transferred to small,

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covered stender dishes containing 20 ml of the following incubation medium: 0.075 M sodium pyrophosphate buffer (pH 8.2) containing 0.1% neotetrazolium chloride and 0.15 M sodium succinate. The tetrazolium and succinate were added to the buffer shortly before use and the freshly prepared medium placed in an incubator. All sections were incubated at 37°C for 45 minutes and then transferred to distilled water. After brief rinsing, they were placed in 10% formalin overnight, washed in water and mounted in glycerine jelly. For comparative purposes we have used also the method described by Seligman and Rutenburg(7). Altogether, over 100 rats have been used in these studies. Kidneys from 10 untreated animals formed the control series while the remaining animals were given injections of either mercuhydrin alone (2-160 mg Hg/kg) or mercuhydrin and one of the following mercurial antagonists: BAL, l-cysteine, or glutathione. The injections were given subcutaneously, except in a very few cases in which the mercuhydrin was given intraperitoneally. BAL (in peanut oil) was administered in a molar ratio of 1:1 of Hg, l-cysteine in molar ratios of 1:1 and 50:1, and glutathione in molar ratios of 1:1 and 10:1. Most of the treated animals have been killed 48 hours after the injection of mercuhydrin since early work indicated that the effect of mercuhydrin was a marked and highly predictable one after this period of time. In addition, the activity of mercuhydrin alone has been studied at the following intervals after its administration: 2, 3, 6, and 24 hours and 2, 3, 4, 6, 7, 10, 11, and 17 days. Finally in a few experiments the *in vitro* effect of mercuhydrin and the mercurial antagonists on sections of normal kidneys was studied.

Histochemical method. We have used neotetrazolium chloride in preference to any of the other tetrazolium salts since its superiority in histochemistry was thoroughly demonstrated by Shelton and Schneider(8). The concentration of pyrophosphate used in this method with *intact cells* does not inhibit the succinoxidase. The method will work equally well, moreover, with 0.1 M phosphate buffer or without any buffer, provided the pH of the medium is properly adjusted prior to incubation. We have been able to show that the effects of mercuhydrin treatment were the same, regardless of whether a phosphate or a pyrophosphate-buffered medium was used. Following the addition of the neotetrazolium and sodium succinate, the pH of the pyrophosphate-buffered medium is approximately 7.3.

Our results with the original method of Seligman and Rutenburg(7) were technically so poor, that we were unable, by their method, to localize the site of mercurial action on the kidney tubule. We used 50 μ sections, as this thickness withstands the necessary manipulations without undue folding, matting, or fragmenting. In such preparations the topographical relationships can be visualized readily and intact tubules can be followed for considerable distances. Sections much thicker than 50 μ are too dense for microscopic study and with sections as thick as 100 μ the method is no longer specific since some coloration occurs in the absence of added substrate. The specificity of the method we have used is shown by the fact that no color develops when succinate is omitted from the medium. Also, we failed to obtain any coloration when sodium malate was substituted for succinate as substrate. Finally, the reaction with added succinate can be inhibited by sodium malonate.

Normal distribution of enzymatic activity. When sections of normal rat kidneys are placed in the described incubation medium, coloration begins in a few minutes and increases progressively for about 30 minutes. The color is pink to purplish, depending on the amount of enzymatic activity, and it results from the intracellular deposits of formazan in the kidney tubules. The glomeruli are essentially uncolored, as are also the collecting ducts. The medulla exhibits three zones: a) an outer, lightly stained area through which pass the descending limbs of Henle's loops, b) an intermediate zone composed of heavily stained parallel limbs of Henle's loops which alternate with radiating unstained columns of collecting ducts and blood capillaries, and c) an inner, essentially unstained, zone which occupies approximately the inner one-half of the medullary area; this is composed of col-

lecting ducts and feebly stained thin segments of Henle's loops (Fig. 1). Our explanation of the sharp boundary which is present between the intermediate and inner zones is not in accord with other interpretations in the recent literature and therefore requires further comment. Mustakallio and Telkkä(6) considered that at this sharp boundary "the dark blue loops of Henle bent to reenter the kidney cortex." In explaining the same phenomenon, Padykula(9) states "the heavily stained mid-region of the medulla contains both loops of Henle and collecting ducts. The staining reaction in the collecting ducts terminates abruptly, forming the innermost mass of pyramid of very faintly stained collecting ducts". We find that neither interpretation is correct—that the sharp boundary results instead from the abrupt transition from thin segments to darkly stained, thick (ascending) limbs of Henle's loops. It is this abrupt transition in the tubules of the juxtamedullary generation of glomeruli which produces the striking zonal boundary as seen in Fig. 5. Padykula's explanation implies that the collecting ducts are stained in the intermediate "mid-region" of the medulla, which is not the case. In our Fig. 1 and 3, unstained columns of collecting ducts are especially well shown in this zone, alternating as they do with areas of heavily stained loops of Henle. The same is clearly illustrated in Fig. 2 of a recent paper by Malaty and Bourne(10). Even in Padykula's own figures (1-4) the collecting ducts in the "mid-region" of the medulla are obviously unstained, although since she presents transverse sections of the kidney, the radiating columns of collecting ducts appear as unstained circular or oval areas. The explanation offered by Mustakallio and Telkkä(6) ignores the presence of any portion of Henle's loops in approximately the inner one-half of the medulla. Since loops can be seen by classical methods in the inner medulla, their explanation seems untenable. Furthermore, during the course of our studies, evidence was obtained from the kidneys of younger animals which clearly shows that the loops of Henle descend deeper into the pyramid than the conspicuous boundary referred to. Thus, in rats younger than 10 days of age, the kidney tubules are relatively undifferentiated and give a uniform coloration in their course through the medulla (Fig. 6). Such loops can readily be observed to approach very near to the apex of the papilla and it is their progressive differentiation during the next 10 days of life which results in the zonal pattern previously described. The convoluted tubules in the cortex are darkly colored in the kidneys of normal animals. This holds for both the proximal and distal convolutions, although the distal tubule is normally somewhat darker than the proximal. Very little variation in the pattern or intensity of coloration in the kidneys of the control series of animals has been observed. As already mentioned, this is in contrast to the extremely inconsistent results we have obtained on a limited number of kidneys using the original method of Seligman and Rutenburg(7).

Mercurial inhibition. Within 2-3 hours following a single, subcutaneous injection of 40 mg Hg/kg of mercuhydrin there is a marked inhibition of the succinic dehydrogenase activity in the *proximal* convoluted tubules. After incubation, the cortex in such sections appears pale on gross examination, and on microscopic examination shows the distal convoluted tubules intensely colored; whereas, the proximal convoluted tubules are now only feebly colored. Even when our highest dose was used (160 mg Hg/kg) neither the complete inhibition of activity, nor a marked inhibition of activity in the thick limb of Henle's loops, as reported by Mustakallio and Telkkä, could be observed. We believe that these differences are due to the inadequacy of their method to demonstrate experimentally induced changes in enzymatic activity. The maximal inhibition of activity observed by us occurred at 24-48 hours following the injection of mercuhydrin (Fig. 2). At this time tubular damage is already apparent, whereas extensive tubular necrosis and cystic degeneration generally result within 3-7 days after the administration of 20 mg Hg/kg (Fig. 4). Higher dosage levels are invariably lethal in the first week after injection, and even a dose of 10 mg Hg/kg is sufficient to cause extensive tubular damage together with marked enzymatic inhibition for at least a week following

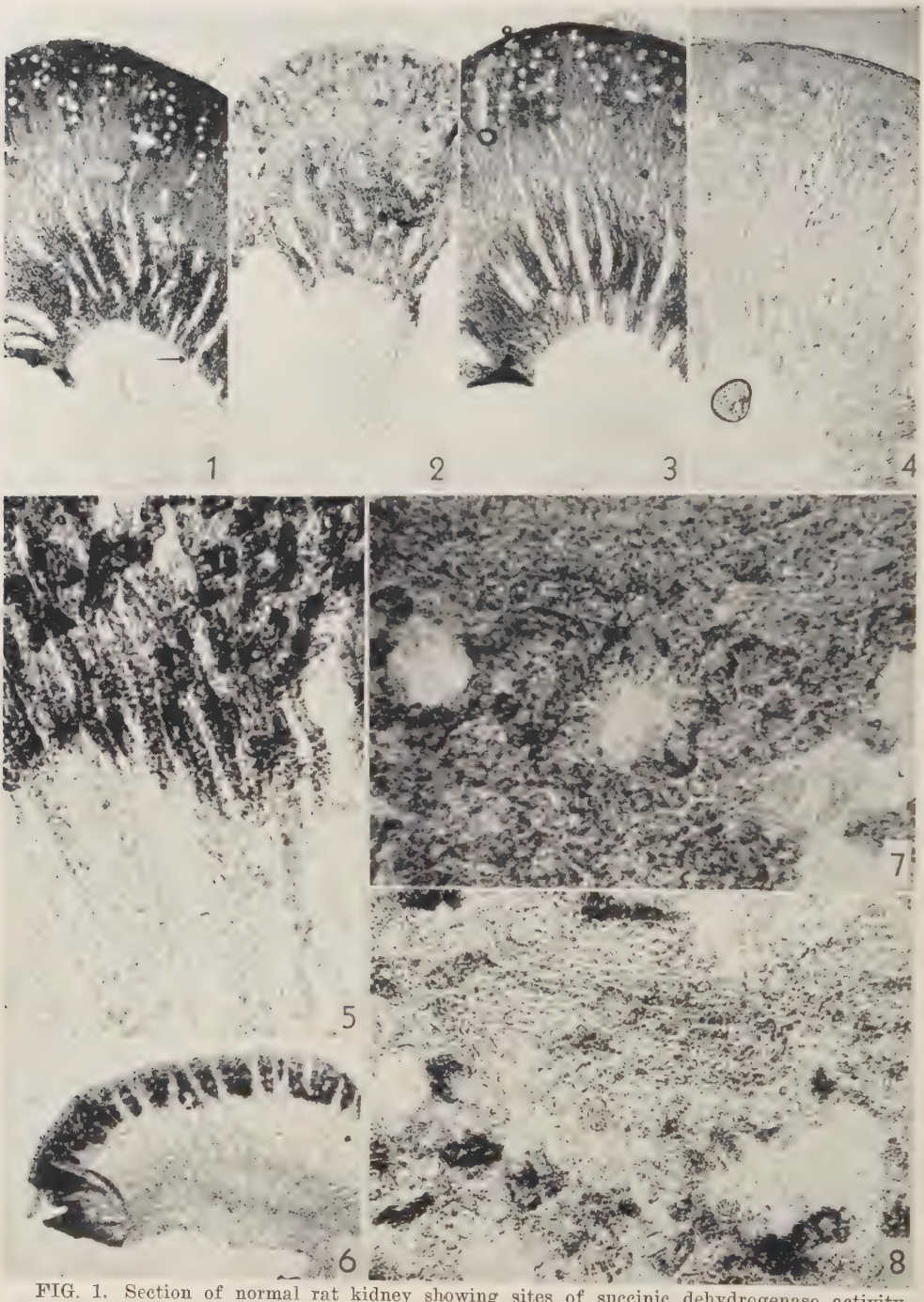

FIG. 1. Section of normal rat kidney showing sites of succinic dehydrogenase activity. Arrow indicates prominent boundary discussed in text. ×9.

FIG. 2. Section of kidney removed 48 hr after subcutaneous injection of 20 mg Hg/kg mercuhydrin. ×9.

FIG. 3. Section of kidney after treatment as described for Fig. 2 except that an equimolar amount of BAL was given simultaneously. ×9.

FIG. 4. Section of kidney removed 7 days after subcutaneous injection of 20 mg Hg/kg of mercuhydrin. (Black circle in lower left is an artifact due to air bubble in preparation.) ×9.

FIG. 5. Higher magnification of area indicated by arrow in Fig. 1. This is the area of abrupt transition from thin segments to thick limbs. ×100.
FIG. 6. Section of 6-day-old normal rat kidney showing undifferentiated tubules. ×12.
FIG. 7. Higher magnification of small area of cortex shown in Fig. 1. ×100.
FIG. 8. Higher magnification of small area of cortex shown in Fig. 2. Reaction is almost completely inhibited in proximal convoluted tubules. ×100.

the injection. We cannot, therefore, agree with Mustakallio and Telkkä in their findings that "The changes after doses of 10-40 mg Hg/kg were reversible within 3 days." We do believe however, that it is unlikely that the very early enzymatic inhibition is due to cellular damage *per se*. It seems more probable that cell injury and necrosis may result from the reduced metabolic capacity of cells which have their sulfhydryl enzymes inactivated. Once tubular necrosis is pronounced, the almost total lack of enzymatic activity must be considered as a secondary result of the cellular damage. Further attempts to correlate the degree of enzymatic inhibition with the histopathology of the kidney after the administration of mercurial agents are now under study.

Up to now we have been unable to demonstrate a marked inhibition of succinic dehydrogenase activity in the kidney with a dose lower than 10 mg Hg/kg. Even with this dosage the effect is not pronounced until some 24-48 hours after the injection. Since larger doses will produce the same effect in a shorter period of time, it seems likely that the effect is dependent on the selective concentration of the mercury in the cells of the proximal convoluted tubules. Virtually the entire amount of mercury contained in a single dose of diuretic is excreted in the urine within 24 to 36 hours(11). This explains the inhibition of succinic dehydrogenase activity *in vivo* only in the kidneys, but not in the heart or liver(3). Actually, there is some reason to believe that mercury is removed from the blood by tubular excretion(12) and if this is the case, one might expect that its passage through the tubular epithelium would result in a selective enzymatic inhibition in that portion of the tubule through which it passes. Pathologists have known for some time that the proximal convoluted tubule is the site of principal damage in cases of mercury poisoning and in experimentally induced mercury damage(13). It is not surprising therefore, to find that this is also the site of maximal enzymatic inhibition. We believe that this observation is of functional significance, inasmuch as we have found that the addition of mercuhydrin (1×10^{-5} M) to the incubation medium results in the total inhibition of the coloration of sections of normal kidneys. Reduction of the titre of mercuhydrin gives progressively less reduction in coloration but without any *differential* effect upon the tubules such as occurs when the mercuhydrin is administered to the animal. This means that the results of enzyme studies on cell homogenates and even on tissue sections cannot be easily interpreted in terms of what happens in the intact organ.

Counteraction of enzyme inhibition. The injection of BAL (in equimolar amounts) simultaneously with an effective dose of mercuhydrin will completely suppress the inhibition of succinic dehydrogenase activity. Sections of kidneys of animals treated in this manner exhibit a normal pattern of enzymatic activity at 48 hours after the injections (Fig. 3). The injection of l-cysteine and glutathione in equimolar and in much larger amounts does not counteract the inhibition when injected simultaneously with mercuhydrin. These two compounds also fail to counteract the diuretic action of mersalyl(4). As in previously reported studies *in vitro*(2), the addition of l-cysteine or glutathione in equimolar amounts, as well as BAL, completely reverses the inhibition. It seems likely that, when injected, monothiols may be oxidized before reaching the kidney.

Summary and conclusions. Mercuhydrin, when administered to rats by subcutaneous injection causes a marked depression of succinic dehydrogenase activity in the proximal convoluted portion of the kidney tubules, while other portions of the nephron are little affected. This enzymatic inhibition is concomitant with the known time of diuresis and both can be prevented by the administration of BAL. This suggests that the enzymatic

inhibition is, in fact, related to the diuresis. This places the locus of mercurial action on the proximal convoluted tubule—most certainly as regards its inhibition of succinic dehydrogenase activity and by implication also its diuretic action.

ADDENDUM: After manuscript was submitted a note appeared in *Science* describing a similar study by Wachstein and Meisel (*Science*, v119, 100). Their findings on localization of mercurial action are in essential agreement with those reported in the present paper.

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Relation of Dietary Protein Levels to Pancreatic Damage in the Rat. (20865)

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Extensive degenerative changes in the pancreas brought about by ethionine, a presumed methionine antagonist(1-3) have focused attention on the influence of dietary factors essential for the structural integrity of the pancreas. While the effects of obstruction of the pancreatic ducts as well as of other mechanical factors which are thought to be of importance in the etiology of human pancreatic necrosis have been extensively investigated(4), few workers have paid attention to the influence of nutrition on the pancreas. The feeding of diets low in protein and/or of poor quality and often high in fat was found to lead to pancreatic damage in the dog(5) and rat(6-9). In ducklings, a synthetic diet rich in sucrose often leads to pancreatic fibrosis(10).

It is the purpose of the present communication to describe briefly the changes that occur in the pancreas of the rat on a synthetic diet devoid of any protein as well as the influence of various protein levels and of methionine in such a synthetic diet on the microscopic structure of the pancreas.

Material and method. Young male rats of the Wistar strain weighing approximately 150 g were used. Sixty animals were given a basic protein free diet. Groups of 15 rats were fed the same diet supplemented with 2, 4, and 7.5% vitamin free test casein (Nutritional Biochemical Corp.) and 1% dl-methionine respectively. The basic diet contained 70% corn starch, 10% vegetable oil, 15% cellulose, 1% cod-liver oil and 4% salt mixture U.S.P. No. XIV. The following vitamin supplements were added per kg of food: Vitamin A concentrate 20,000 units, Viosterol 11,000 units, inositol 110 mg, alpha tocopherol 110 mg, choline chloride 1.65 g, Menadione 49 mg, para-amino benzoic acid 110 mg, niacin 100 mg, riboflavin 22 mg, pyridoxine 22 mg, thiamine 22 mg, calcium pantothenate 66 mg, biotin 0.44 mg, folic acid 2 mg, vit. B_{12} 0.03 mg. Animals on the basic protein free diet were allowed to live up to 75 days in order to study the development of the pancreatic lesions. Rats were sacrificed on the 5th, 7th,

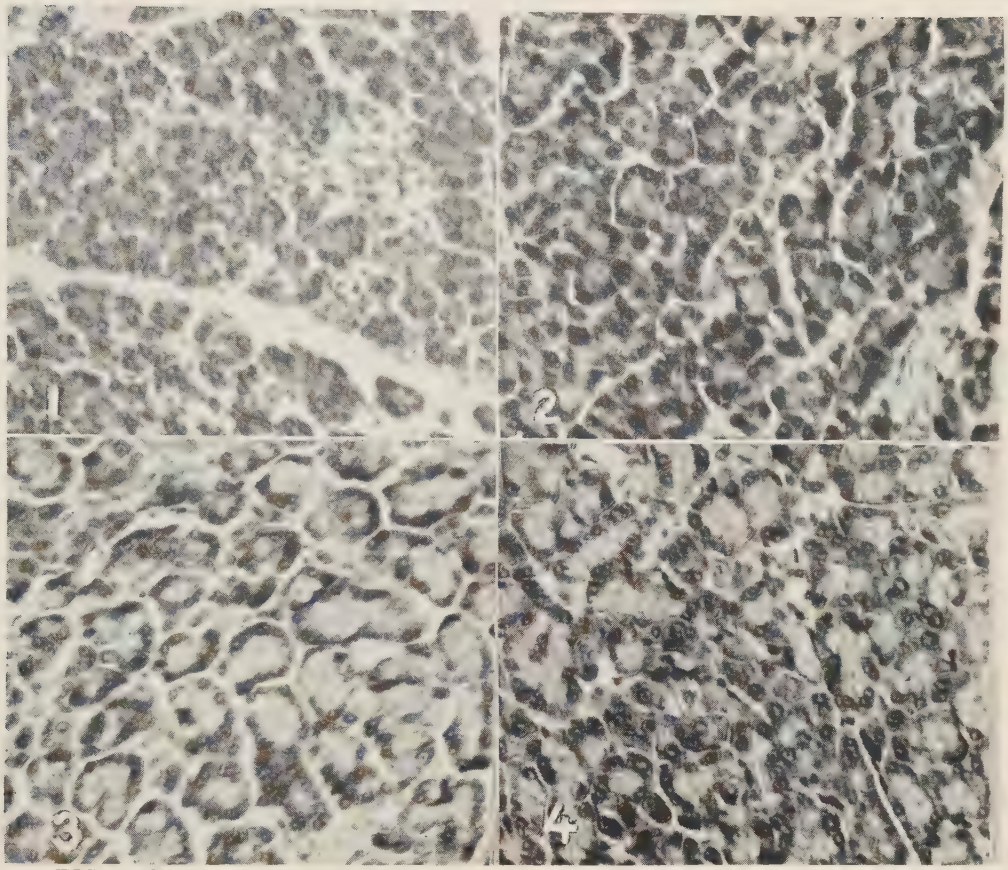

FIG. 1. Section of pancreas from a rat on the basic protein free diet for 45 days. Note marked atrophy of cells, disassociation of acini and focal areas of fibrosis. Hematoxylin-eosin × 300.

FIG. 2. Section of pancreas from a rat on the basic protein free diet supplemented with 4% casein, for 45 days. Atrophy of acinar cells and fibrosis is considerably less marked. Hematoxylin-eosin × 300.

FIG. 3. Section of pancreas from a rat on the basic protein free diet supplemented with 7.5% casein, for 45 days. The microscopic appearance of the acinar cells is almost normal. Hematoxylin-eosin × 300.

FIG. 4. Section of pancreas from a rat on the basic protein free diet supplemented with 1% dl-methionine, for 45 days. The microscopic appearance of the pancreas is similar to that depicted in Fig. 2. Hematoxylin-eosin × 300.

11th, 14th, 18th, 26th, 35th, 40th, 45th, and 75th day. Rats that died spontaneously in this or the other groups were not used. For the evaluation of the protein supplements the rats were kept for 45 days since within this time pancreatic damage in rats on the basic diet was quite marked. At the time of sacrifice pieces of liver, kidneys and pancreas were removed, fixed in Bouin's solution and stained with hematoxylin-eosin. In most cases sections were also stained with Gomori's chrome alum hematoxylin method. Other special stains were used when indicated.

Results. On the basic diet, changes developed after 10 to 12 days. There was seen gradual disappearance of zymogen granules and diminution in the size of acinar cells. Within the next weeks these changes became more pronounced. In rats sacrificed after 40 to 45 days the pancreas revealed marked atrophy of the acini (Fig. 1). Most of the zymogen granules were absent and many acinar cells consisted of nuclei with moderately prominent nucleoli which were surrounded by a markedly reduced amount of basophilic cytoplasm. Occasional cells appeared to consist of almost

naked nuclei. Due to the atrophy of the excretory cells the acini became dissociated from each other and were apparently separated by empty spaces. There was focal proliferation of fibroblastic cells which were surrounded by fine strands of connective tissue. There was only scanty infiltration by mononuclear cells. Some of the smaller ducts were dilated, but no cyst formation was noticed. In animals which were permitted to live for 75 days these changes became still more pronounced. By then almost every microscopic field showed evidence of marked atrophy of acinar cells with subsequent loss of the normal pancreatic architecture and focal connective tissue proliferation. There was, however, no change in the Langerhans islands and there was no degranulation of alpha or beta cells.

In the liver of animals which had been on the diet for 12 days the typical changes of protein depletion as described by Elman, Smith and Sachar(11) were seen. The liver cells appeared enlarged with prominent cell walls and rarified cytoplasm with only little stainable fat. In other livers, however, especially in rats which were on the diet for long periods of time, the liver cells showed considerable fatty infiltration. The average weight loss in 12 rats after 45 days was 67 g.

Basic diet plus 2% casein. The changes in the pancreas were as pronounced as on the completely protein free diet. Atrophy of single cells and dissociation of the acinar pattern was quite uniform. In many microscopic fields early fibrosis was noted. The liver cells were small and contained various amounts of stainable fat. The average weight loss of 7 rats in 45 days was 60 g.

Basic diet plus 4% casein. Pancreatic changes were considerably milder (Fig. 2). Many of the acinar cells contained zymogen granules and an almost normal amount of basophilic cytoplasm. Consequently the acinar pattern was considerably less disturbed than in the previously described two groups. Fibrosis was very scanty. The livers showed small cells with only traces of stainable fat. The average weight loss of 10 animals in 45 days was 42 g.

Basic diet plus 7.5% casein. The pancreas of these animals appeared almost normal (Fig. 3). The acini consisted of good sized cells with distinct perinuclear basophilia and well developed zymogen granules. Only occasional acini mostly in the vicinity of Langerhans islands showed a somewhat swollen cytoplasm with prominent zymogen granules and slightly decreased amounts of perinuclear basophilia. The liver cells appeared normal and contained only occasional traces of stainable fat. The average weight loss of 10 animals in 45 days was 3.5 g.

Basic diet plus 1% methionine. As in the group supplemented with 4% casein there was considerable protection of the pancreatic structure (Fig. 4). Most acinar cells showed perinuclear basophilia and a normal amount of zymogen granules. Atrophic cells and dissociated acini were only occasionally seen and there was no appreciable fibrosis. Liver cells were small without stainable fat. The average weight loss of 7 animals in 45 days was 56 g.

Comment. The basic changes seen in the pancreas on a completely protein deficient diet supplemented with the necessary vitamins consisted essentially of a slowly developing atrophy of acinar cells with loss of zymogen granules, with subsequent disassociation of acini and distortion of the normal architectural pattern. There was almost no inflammatory reaction but focal replacement fibrosis commenced after several weeks and became very marked within 75 days. Cystic degeneration as described by Kristal(8) and Véghelyi and coworkers(9) was not seen. It should, however, be noted that these investigators used diets containing various kinds of nutritionally inadequate proteins, often incomplete vitamin supplementation, as well as longer experimental periods(8).

Supplementation of the basic protein free diet with 2% casein was without any effect. Supplementation with 4% casein, however, was of considerable benefit. On a diet containing 7.5% casein the pancreas showed an almost normal microscopic pattern. It is interesting to note that at least 6% casein is necessary for the normal reproductive function of the rat. Pregnancies in rats given smaller amounts of casein terminated in reabsorption and abortion(12).

The considerable protective action of methionine described in this communication confirms and enlarges observations previously made by Véghelyi and his co-workers(9). The weight loss of animals on a methionine supplemented diet was of almost the same magnitude as that on the basic protein free diet with or without supplementation with 2% casein.

The changes on the basic protein free diet differ markedly from those induced by ethionine. The atrophic lesions induced by protein deficiency developed much slower. There is complete absence of any necrosis of acinar cells. Moreover the acinar cells do not show loss of perinuclear basophilia and de-differentiation which make them resemble fibroblasts (3). There is also a considerable difference in the effect on the liver since in protein deficiency necrosis of liver cells with subsequent diffuse fibrosis which follows the ingestion of methionine(13,14), is not observed. These considerable differences suggest that the effect of ethionine is probably not due to a conditioned methionine deficiency only, but is rather of a considerably more complex nature.

Summary. The development of typical atrophic lesions of the pancreas in the rat on a completely protein free diet is described. Supplementation of such a diet with 2% casein is without influence on the pancreas, while either 4% casein or 1% methionine have a marked beneficial effect. On a synthetic diet containing 7.5% casein the microscopic appearance of the pancreas is almost normal.

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Conversion of C^{14} Carotene to a Non-Saponifiable Substance or Substances in the Rat.* (20866)

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The conversion of carotene to vitamin A is a well established process; however, it remains to be demonstrated if this compound is a precursor for other body constituents. The object of this investigation was to determine if a part of the absorbed carotene was converted to some substance or substances in a carotene-free, non-saponifiable fraction in different tissues of the rat.

Method. A sample of uniformly labelled C^{14} carotene (90% beta)† having a specific activity of 0.4 microcurie per milligram, a total activity of 1.5 microcuries, and 4900 counts per minute was suspended in 3 ml of

* This work was supported in part by a grant from National Vitamin Foundation.

† The carotene was purchased from Nuclear Instrument and Chemical Corp., Chicago, Ill.

water using Tween 80 as the emulsifier and fed by a stomach tube to a male, white rat weighing about 200 g. The rat was sacrificed by decapitation 24 hours later. A second rat weighing approximately the same was given a similarly prepared dose of carotene having 2100 counts per minute. This animal was sacrificed at the end of 6 hours. The non-saponifiable substances were extracted from feces, liver, small intestine, and extrahepato-intestinal tissue according to the method of Hjarde(1). Liver, small intestine, and extra-hepato-intestinal tissue extracts were freed from carotene by the chromatographic procedures of Glover *et al.*(2). No carotene was demonstrated in these extracts when they were analyzed in a Beckman Spectrophotometer at 450 mμ. Throughout this paper, the carotene-free, non-saponifiable fraction will be referred to as the CFNS fraction. The carotene content of the non-saponifiable fraction from feces was measured spectrophotometrically without separating it from the extract. The radioactivity of the dry extracts was measured under an end window Geiger-Muller tube. The vit. A of the CFNS fractions was determined colorimetrically using $SbCl_3$ according to the technic of Glover *et al.*(2). The extinction of the petroleum ether solutions was measured at 450 mμ and accepting the $E^{1\%}_{1\ cm}$ for beta-carotene as 2500, the amount of carotene present was calculated.

Results. Table I summarizes the results of this investigation. The 24-hour animal absorbed approximately 30% of the total C^{14} fed. The remaining 70% of the administered dose remained in the feces. The CFNS extracts of liver, small intestine, and extra-hepato-intestinal tissue were found to contain respectively 4.4, 6.1, and 20.0% of the C^{14} fed. The 6-hour animal absorbed approximately 54% of the isotope fed. In the liver, intestine, and extrahepato-intestinal tissues there was found 6.7, 14.1, and 33.3%, respectively, of the dose administered. If the amounts of C^{14} found in the extracts of the liver, intestine, and extrahepato-intestinal tissues were calculated in terms of the per cent of dose absorbed, then the tissues of the twenty-four-hour animal contained 14.4, 20.1,

TABLE I. Isotope Concentration in Carotene-Free Non-Saponifiable Fractions of Rats Fed C^{14} Labelled Carotene.

The 24-hr animal was fed 3.75 mg carotene containing 4900 c.p.m. of uniformly labelled C^{14}. The 6-hr animal was fed 1.56 mg carotene containing 2100 c.p.m. of uniformly labelled C^{14}.

Fraction isolated	C^{14} found, c.p.m.	% of total dose fed*
24-hr animal		
Carotene-free non-saponifiable		
Liver	216	4.4
Small intestine	301	6.1
Extrahepatointestinal tissue	980	20.0
Total non-saponifiable		
Feces	3500	71.0
6-hr animal		
Carotene-free non-saponifiable		
Liver	141	6.7
Small intestine	297	14.1
Extrahepatointestinal tissue	700	33.0
Total non-saponifiable		
Feces	900	42.7

* $\dfrac{\text{C.p.m. in fraction isolated}}{\text{C.p.m. in carotene fed}} \times 100.$

and 65.5% and those of the 6-hr animal had 12.4, 26.0, and 61.5% respectively.

The content of vit. A found in liver, intestine, and extrahepato-intestinal tissue after feeding the carotene was essentially similar for both the 24- and 6-hr rats, namely, approximately 1000 μg, 30 μg, and 90 μg, respectively. The carotene content of the non-saponifiable fraction from the feces of the 24-hr rat was 2.64 μg and 0.69 μg for the 6-hr animal.

Discussion. Since the combined CFNS tissue extracts from each animal accounted for approximately 100% of the absorbed C^{14}, one may infer that carotene is converted to some substance or substances found in the non-saponifiable fraction and is not changed into any water soluble or saponifiable material.

With the procedure used in this experiment it was not possible to demonstrate that vit. A was the only active substance found in this fraction. It is interesting to note that as soon as 6 hours after feeding the C^{14} labelled carotene, the highest percentage of C^{14} was found in tissues outside the liver and small intestine, thus indicating that the conversion product or products of carotene are rapidly

distributed throughout the body. Evidence has been presented to indicate that the intestine is a site where carotene is converted to vit. A(2). The finding that the uptake of C^{14} in the CFNS fraction was higher at the end of 6 hours than after 24 hours may be considered as presumptive evidence that the small intestine is one of the sites for this conversion process.

Summary. The feeding of C^{14} labelled carotene resulted in the deposition of all of the absorbed C^{14} in the carotene-free, non-saponifiable fraction from the total animal. The highest concentration of C^{14} was found in the extrahepato-intestinal tissues, and the least amount was recovered in the liver.

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Congenital Malformations Produced by Injecting Azo Blue into Pregnant Rats.* (20867)

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Gillman and his coworkers(1) have shown that the injection of trypan blue into female rats before and during pregnancy causes maldevelopment in the offspring. A single dose of 1 cc of 1% aqueous solution during the first 9 days of pregnancy caused anomalies in 8.6% of the young, but when treatment was given before as well as during pregnancy, 32.4% of the young were congenitally defective.

As an extension of this significant observation the present author has undertaken to study the teratogenic activity of other azo compounds more or less closely related to trypan blue. The original experimental procedure of Gillman and collaborators has been simplified and standardized to facilitate a comparison of the results of trypan blue with those of other compounds tested. One cc of 1% aqueous solution was injected subcutaneously into pregnant rats on the 7th, 8th and 9th days of gestation. (Pregnancy was considered to be of 1 day's duration 24 hours after sperm were found in the vaginal smear.) On the first day of treatment (7th day of gestation) the females were laparotomized to verify pregnancy and to determine the number of implantation sites. Control females were similarly laparotomized and injected with distilled water during pregnancy. No further treatment was given after the last injection on the 9th day. The animals were killed on the 20th day, one day prior to expected delivery, in order that living fetuses as well as resorption sites might be observed *in situ*. The living offspring were removed, weighed and examined externally for malformations. They were then preserved in Bouin's fluid for later dissection or histologic sectioning. Dissection consisted of making serial transverse slices with a razor blade at 1-2 mm intervals through the head and trunk. The slices were examined and further dismembered, if necessary, under a dissecting microscope. This made possible the recognition of a wide range of visceral and other internal malformations without the tedium of preparing and studying serial histologic sections.

Results. Of the compounds tested to date, azo blue has proven the most effective in producing developmental abnormalities. Its effects on both pregnancy and on the offspring are summarized and compared in Table I with the effects of trypan blue tested under similar conditions. The data suggest that azo blue is less likely than trypan blue to cause early termination of pregnancy as a result of ma-

* This investigation was supported by a research grant from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service.

TABLE I. Effects on Gestation and on Offspring of Azo Blue and Trypan Blue Injected in 1% Aqueous Solution on the 7th, 8th and 9th Days of Pregnancy.

		Pregnancies terminating before 20th day		Pregnancies continuing to 20th day			
Treatment	No. of mothers	% maternal death	% complete resorption	%	No. implantations	% dead or resorbed	% survivors malformed
Azo blue	13	0	15	85	134	53	59
Trypan blue	20	10	25	65	151	38	49
Control	6	0	0	100	57	9	0

ternal death or complete resorption, but more likely to cause abnormal development of the offspring. Although the validity of these differences cannot be established until larger numbers of animals have been accumulated, there is no doubt that azo blue is a potent teratogenic agent.

The types of malformations, as well as their incidence, were generally similar following treatment with azo blue or trypan blue (Table II). Despite a dissimilar incidence of cardiovascular, genitourinary and appendicular defects, probably attributable to the small number of cases, the pattern of abnormality appears to be essentially the same after treatment with either dye. The pattern is also approximately that described by Gillman *et al.* for trypan blue except that these authors did not report cardiovascular and genitourinary anomalies.

The most frequent specific defect after treatment with either azo blue or trypan blue was hydrocephalus. This condition was not always readily recognizable on external examination of the 20-day fetus but was clearly visible in all degrees when razor-blade slices were made through the brain region (Fig. 2 and 3). Several other malformations, however, were conspicuous on external examination. The trunk was often seen to be shortened due to reduction of the number of vertebrae in the lumbar and sacral regions, and in many of these instances the tail was entirely missing (Fig. 5). Sometimes associated with shortened trunk were anal atresia, absent genital tubercle and urethral orifice, or

TABLE II. Frequency of Malformations in Various Organs and Regions (Expressed as % of Total Living Offspring).

Treatment	Brain	Eye	Vertebral column	Cardiovascular	Urogenital	Gastroschisis	Appendicular	Other
Azo blue	29	14	19	16	5	5	1	4
Trypan blue	34	12	22	3	1	3	4	5

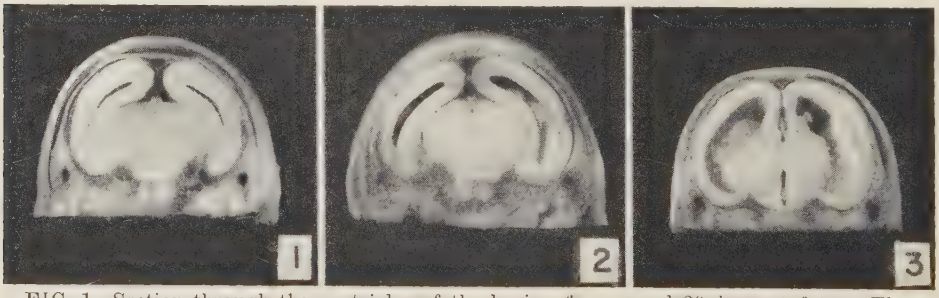


FIG. 1. Section through the ventricles of the brain of a normal 20-day rat fetus. The section was made free-hand with a razor blade.

FIG. 2. Mild degree of hydrocephalus in the lateral ventricles of the brain of a 20-day fetus from a mother injected with azo blue. This degree of hydrocephalus was not recognizable in the *intact* animal.

FIG. 3. Advanced hydrocephalus in the lateral ventricles of the brain of a 20-day fetus from a mother injected with azo blue. The 3rd ventricle is mildly distended.

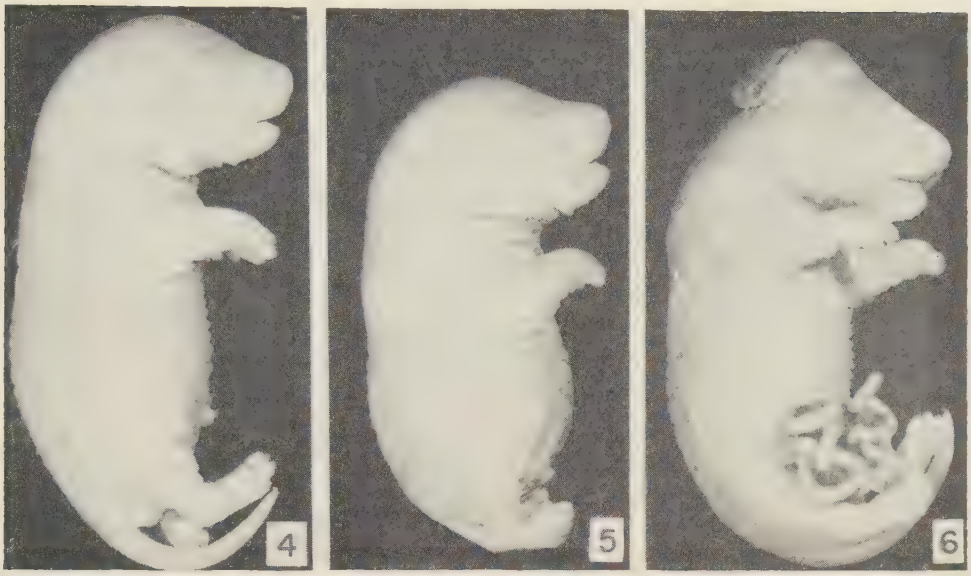


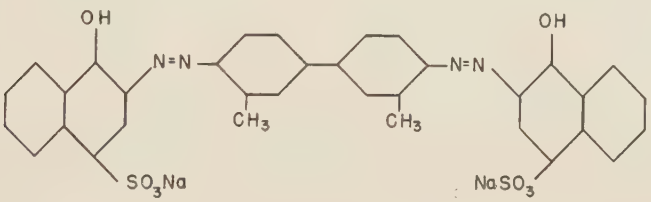
FIG. 4. Normal 20-day rat fetus from a mother injected with distilled water.

FIG. 5. Shortening of the vertebral column with concomitant shortening of the trunk in a 20-day fetus from a mother injected with azo blue. The tail and genital tubercle were missing and the anus and urethral orifices were atretic. The hind limbs were directed more toward the caudal than the ventral aspect of the body.

FIG. 6. Exencephaly, anophthalmia and gastroschisis in a 20-day fetus from a mother injected with azo blue. These particular defects did not regularly occur together.

displaced hind limbs. Also easily detected externally, but somewhat less frequent in occurrence, were exencephaly (Fig. 6), meningocele, spina bifida, gastrochisis (Fig. 6), and clubbed feet. Anophthalmia was visible externally (Fig. 6), but other ocular abnormali-

AZO BLUE (c.i. 463)



TRYPAN BLUE (c.i. 477)

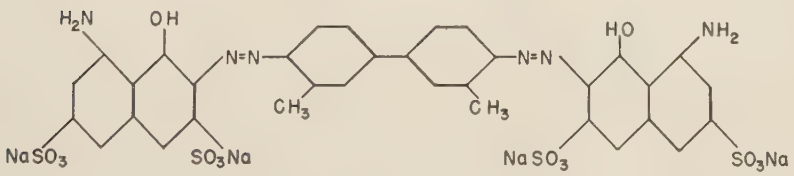


FIG. 7. Structural formula of azo blue and trypan blue. It is noteworthy that both compounds possess the diazotized ortho-tolidine grouping 

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ties such as microphthalmia and colaboma were identified positively only in dissections or histologic preparations. The latter technics were also required to identify cardiovascular and internal genitourinary defects.

Comment. All azo dyes do not possess teratogenic properties. Gillman and collaborators have tested trypan red, Sudan IV, Bismarck brown and Niagara blue and found these compounds inactive as teratogenic agents. The present author has tested Niagara blue 4B and Congo red and likewise found both ineffective (unpublished data). Although several of these are closely related to trypan blue and azo blue, it is probably significant that of the substances tested only the latter two contain the diazotized ortho-tolidine group (3,3′-dimethyl-4,4′ diazobiphenyl) (Fig. 7).

Summary. The injection of 1 cc of a 1% aqueous solution of the diazo dye azo blue into female rats on the 7th, 8th and 9th days of pregnancy resulted in congenital malformations in 59% of living fetuses removed on the 20th day of gestation. The incidence was higher than that obtained with trypan blue (49%) under the same conditions, but the types of malformations were similar.

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Reagents Modifying Stimulating Action of 2, 4 Dinitrophenol.* (20868)

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That 2-4 dinitrophenol (DNP) stimulates endogenous O_2 uptake and inhibits phosphorylation in living intact cells and their homogenates seems fairly well established(1). A wide variety of biological materials has been employed in arriving at such a conclusion. Certain differences in reaction of *intact cells* and their *homogenates* to the reagent, however, suggest the possibility of additional mechanisms entering into the above reactions. The present results have to do with a study of the action of DNP on the endogenous O_2 uptake of intact embryonic cells of the grasshopper (*Melanoplus differentialis*) and the effects of various reagents on such a reaction.

As previously shown from this laboratory (2), mitotically blocked intact embryonic cells respond to a greater degree than similar mitotically active ones. Such differential reactions to the reagent have also been pointed out for other tissues showing different degrees of metabolic activity(1). It has also been shown for intact embryos of the grasshopper that the concentration (5×10^{-5} M) of DNP producing maximal stimulation of endogenous O_2 uptake when added to the homogenate of the embryos produces no change whatever in its endogenous O_2 uptake. A somewhat similar situation has also been reported for the ova of the sea urchin(3). Since detergents are highly surface active compounds and have also been suggested to interfere with phosphorylation phenomena(1) it seemed desirable to determine their effects on the endogenous O_2 uptake of embryos and homogenates and also to see to what extent they might modify the stimulating action of DNP.

Results. In Table I are shown data for the effect of duponol and aerosol OT on the stimulating action of DNP on the endogenous O_2 uptake of intact diapause embryos. An inspection of this table shows that both detergents in appropriate concentrations completely antagonize the stimulating action of DNP. Determinations of phosphates have been carried out by the method of Sumner(4) and it is of some interest to note differences in the reactions of DNP and detergents on the labile phosphorus. With concentrations of DNP (5×10^{-5} M) the labile phosphorus

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TABLE I. Effect of Detergents (Duponol, Aerosol OT) on Stimulation of Endogenous O_2 Uptake of Intact Embryos Due to DNP (Diapause Embryos in Ringer).

DNP	Duponol, %	% of control O_2
5×10^{-5} M	—	196
"	.01	172
"	.05*	100
"	.10	60
—	.05	100
	Aerosol OT	
5×10^{-5} M	—	196
"	.002	196
"	.020*	100
"	.10	49
—	.020	100

* Concentration completely antagonizing stimulating effects of DNP.

(10-minute hydrolysis) of the intact diapause embryo drops to approximately 30% of the untreated control. Such a result indicates that in this reaction of DNP phosphorylation is markedly interfered with. Concentrations of 0.025% aerosol OT which completely antagonize the stimulating action of DNP cause no appreciable change in labile phosphorus of the intact embryo. It would seem, therefore, that perhaps some surface activity of the detergents seriously interferes with the stimulating action of DNP on the intact embryonic cells.

Other reagents and conditions which might possibly affect the stimulating action of DNP on the intact embryo have also been tested. Among them the following produced no effects: B.A.L., methylene blue, succinate, fructose, 1.6 diphosphate, ATP. Subjection of the intact embryo to high centrifugal forces so as to stratify without beaking the cells produced no change in the stimulating action of DNP. Intact embryos soaked in 5×10^{-5} M DNP for 1½ hours, then washed 3× in Ringer, show no effects of the DNP on their endogenous O_2 uptake. Homogenates of embryos in DNP when added to intact embryos produce stimulation of O_2 uptake similar to that by DNP alone. Apparently DNP is held loosely by the embryonic cells and can readily be washed out.

In *summary,* one might reasonably assume that in addition to the stimulating action of DNP and its effect upon the phosphorylating system of the intact living embryonic cell, surface reactions may also play an important part in its reactions.

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Effect of O-Methoxyphenylglycerol Ether (Resyl) on Spinal Reflex Arcs. (20869)

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(With the technical assistance of Robert Moore.)

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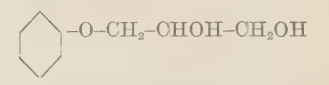
Recent studies indicated that o-methoxyphenylglycerol ether (Resyl) is of specific therapeutic value in the treatment of tetanus and as a muscle relaxant in anesthesia(1-6). This guaiacol derivative is chemically and pharmacologically related to Myanesin(2). However, according to Ginzel, Tschabitscher, and Wayand(5) it has less hemolytic effects than Myanesin in therapeutic doses, which is considered to be an advantage in its clinical use. An electrophysiological

Resyl: 

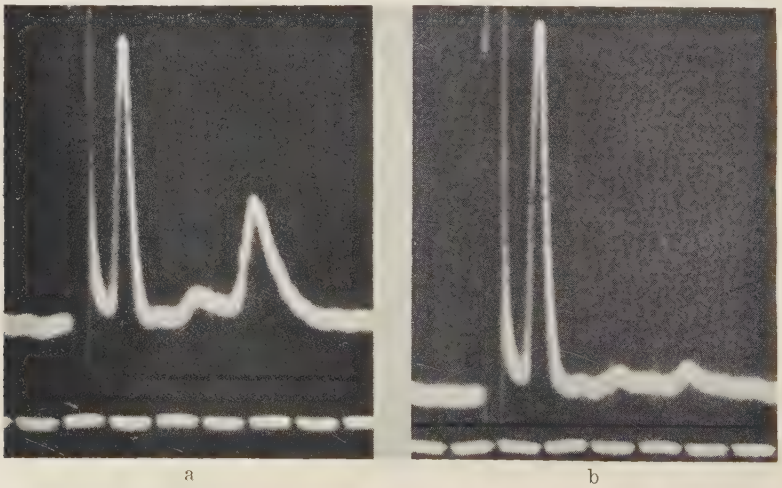
evaluation of the action of Resyl on spinal reflexes was conducted in view of the apparent

TABLE I. Comparison of Equally Effective Doses of Resyl and Myanesin after Intravenous Administration on a Polysynaptic Reflex Arc in the Cat. The reduction in amplitude of contraction of the anterior tibialis muscle was taken as a criterion. Note that 3 times the dose of Resyl was necessary to cause approximately the same quantitative effect as Myanesin.

Drug	Cat	Date	Stimulus (6/min.) Volts	Duration, ms.	Dose, mg/kg	Dose ratio	Reduced amplitude of contraction of tibialis ant. muscle, %	Avg reduction of amplitude, %
Resyl	F	10/23	1.5	1.0	75	3	90	71.5
	M	11/5	.7	1.6	75		53	
Myanesin	F	11/4	.3	.1	25	1	93	77
	F	11/4	1.2	.1	25		79	
	M	11/5	.7	1.5	25		59	

clinical value of the drug.

Methods and materials. Ten cats weighing 2-3.5 kg were employed in this study. Dial in a dose of 70 mg/kg i.p. was used as an anesthetic in 3 of the animals. Five of the cats were decerebrated and 2 were pithed under ether anesthesia, with the ether being discontinued after destruction of the brain. The pithed animals were maintained under artificial respiration. The *effect of Resyl and Myanesin* on a polysynaptic reflex arc was tested by studying the action of these preparations upon the contractions of the anterior tibialis muscle after stimulation of homolateral afferent fibers in the cut tibial portion of the sciatic nerve. Single square wave impulses (0.6-1.2 vs; 0.11-1.0 ms; 6/min.) were applied. In two of these experiments the contralateral peroneal nerve was prepared, cut centrally and stimulated, thus eliminating the reflex arc. The blood pressure was recorded with a mercury manometer. In a second group of experiments the spinal cord of cats was prepared and both dorsal and ventral roots, usually of the S_1 segment, were cut on one side. The whole area was then submerged in mineral oil. Two platinum electrodes were applied to the dorsal root which was stimulated by single square wave impulses (0.4-1.2 vs; 0.03 ms; 4/min.). Action potentials were recorded from the corresponding ventral root with platinum electrodes. The action po-



a b

FIG. 1. Cat, decerebrated, 2.2 kg, F. Oscillographic record of action potentials taken from a ventral root of a cat spinal cord after dorsal root stimulation with single square wave impulses (0.8 v., 0.01 ms., 4/sec.). Time interval 2 ms. One and eight-tenths millisecond following the onset of the stimulus a monosynaptic spike occurred. Following the monosynaptic spike (latency 4.5-12 ms.) the polysynaptic potential presented itself. a. Control. b. 4 min. after intravenous injections of 75 mg/kg of Resyl. Note the disappearance of the polysynaptic spikes in b.

TABLE II. Effect of Resyl on Height of Monosynaptic and Polysynaptic Responses to Dorsal Root Stimulation in the Cat. Frequency/min., 4. The reduction of the height of the polysynaptic spikes after 75 mg/kg i.v. was statistically significant ($P>0.01$). For comparison 2 experiments with Myanesin were tabulated.

				Stimulus (4/min.)		Ht of potentials before drug		Ht of potentials after drug			Effect			Recovery of polysynaptic response		
Date	Anesthesia	Drug	i.v. dose, mg/kg	Volts	Duration, ms.	Monosynaptic, mm	Polysynaptic, mm	Min. after drug	Monosynaptic, mm	Polysynaptic, mm	Monosynaptic, mm	Polysynaptic, mm	Polysynaptic, %	mm	%	In min.
7/21/53	Dial, 0.07 g/kg	R	50	.5	.03	16	4	4	16	.5	0	—3.5	— 87	2	50	85
9/ 2	D*	R	50	.5	"	18	7	3	18	5	0	—2	— 28	7	100	25
9/ 1	D	R	50	.8	"	28	7	4	27	5	—1	—2	— 28	6	86	25
7/15	Dial, 0.07 g/kg	R	75	.8	"	10	12	7	6	2	—4	—10	— 83	3	35	100
9/ 2	D	R	75	.5	"	18	6	2	14	0	—4	—6	—100	5	83	55
9/28	D	R	75	.8	.01	13	6	4	18	4	+5	—2	— 33	6	100	55
10/ 2	D	R	75	1.1	"	14	5	6	22	1	+8	—4	— 80	4	75	45
10/16	D	R	75	.9	"	11	4	4	15	1	+4	—3	— 75	3	75	55
9/ 1	D	M	50	.8	.03	20	9	11	7	0	—9	—2	—100	8	72	55
9/ 2	D	M	50	.5	"	16	5.5	2	10	0	—6	—5.5	—100	4	73	50

* D = Decerebration; R = Resyl; M = Myanesin.

tentials were amplified by a DC preamplifier and fed into the amplifier of a Dumont double beam oscilloscope and photographed with a Grass recording camera. *Toxicity* studies were done in rats and the LD_{50} was calculated according to the method of Behrens(7). All injections were *administered intravenously* and doses of Resyl mentioned refer to milligrams of o-methoxyphenylglycerol ether.

Results. The contractions of the tibialis muscle after stimulation of the central end of the cut tibial portion of the sciatic nerve was markedly reduced after doses of 50-75 mg/kg of Resyl. The contractions following stimulation of the peripheral part of the cut peroneal nerve of the contralateral side was not affected by the same doses of Resyl. The injection of Resyl in the doses mentioned above regularly produced a transient fall in blood pressure. A comparison of the effect of Resyl and Myanesin indicated that the latter is about 3 times more active on a milligram per milligram basis (Table I).

The action potentials recorded from the ventral roots after stimulation of the corresponding dorsal root showed a typical monosynaptic spike (latency approximately 1.5 ms) which was followed by polysynaptic potentials (latency 2.5-15 ms) (Fig. 1a). The injection of 75 mg/kg of Resyl markedly reduced or completely abolished the polysynaptic responses (Fig. 1b), whereas, the monosynaptic spike was unchanged, slightly decreased or augmented. The height of the polysynaptic potentials was significantly reduced ($P<0.01$) following 75 mg/kg of Resyl.

Fifty mg/kg of Myanesin uniformly abolished the polysynaptic response in contrast to the same dose of Resyl which failed to produce a significant reduction ($P>0.1$).

The results of these studies are summarized in Table II.

In some of the experiments the height of the monosynaptic spike was increased after Resyl while in others a definite decrease was observed. However, these changes were not statistically significant.

The toxic manifestations of both Resyl and Myanesin in rats after intravenous injections consisted of dyspnea, ataxia, paralysis and hematuria. The incidence of hematuria was greater after toxic doses of Myanesin than after Resyl. The calculated LD_{50} for Resyl was 360 mg/kg as compared to 133 mg/kg for Myanesin.

Discussion. These results show that the clinical findings of Ginzel and Wayand(3,4),

Ginzel, Tschabitscher, and Wayand(5) and of Bowers(6) with o-methoxyphenylglycerol ether in the treatment of tetanus could be explained on the basis of inhibition of polysynaptic reflexes. These authors found that side reactions such as hemolysis, anemia and hemoglobinuria which are frequently produced by the i.v. injection of Myanesin were not produced by the i.v. administration of o-methoxyphenylglycerol ether, although the same well-known pharmacological effects on spinal reflexes described for Myanesin by Berger and Bradley(8) and by Henneman, Kaplan and Unna(9) could be obtained.

It can be stated with certainty that Myanesin is the more potent drug on a milligram per milligram basis, its potency being probably about 3 times that of Resyl. On the other hand the LD_{50} of Myanesin in rats is about 3 times lower than the LD_{50} of Resyl. Although higher doses of Resyl are required to obtain quantitatively equal effects as with Myanesin the therapeutic ratio of both drugs is about the same.

Summary. 1. Resyl (o-methoxyphenylglycerol ether) has been found to block effectively the transmission of impulses through the internuncial units of spinal reflex arcs in cats in doses of 75 mg/kg. 2. On a mg/mg basis Myanesin is about 3 times more active than Resyl, however, on the basis of the ratio of activity to toxicity Resyl and Myanesin appeared to be equally effective.

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Effect of Carboxylic and Amino Acids on Fibrinolysis Produced by Plasmin, Plasminogen Activator and Proteases.* (20870)

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It was reported recently that citrate increases the fibrinolysis induced by streptokinase-activated human globulin(1). This agent(2) and the globulin fraction isolated from spontaneously active human blood(3) were later found to be potent activators of plasminogen. Both preparations produce fibrinolysis chiefly by activation of the plasminogen present in the fibrin substrate. In the present paper bovine plasmin and a number of other proteases were compared with the plasminogen activators to determine whether the increased fibrinolysis depends on a modified enzymatic effect on fibrin or on activation of plasminogen in the substrate. A number of chemical compounds were studied in an effort to find a possible relationship between increased fibrinolysis and chemical structure.

* Supported by a grant from the Josiah Macy, Jr. Foundation, New York, to Dr. Tage Astrup.

Methods and materials. The following plasminogen activator preparations were used: *Streptokinase activated globulin* (*"S-activator"*): Normal human globulin was activated by streptokinase and subsequently lyophilized(4). Buffer solutions were prepared, in which the globulin concentration was 1/20 of the original concentration in serum. *Spontaneously active globulin* (*"Spontaneous activator"*): A lyophilized globulin fraction was prepared from spontaneously active blood obtained from human corpses after sudden anoxaemic death(5). Buffer solutions were

TABLE I. Activity of Fibrinolytic Agents on Fibrin Containing Salts of Carboxylic and Amino Acids.

Agent	Preparation	Fibrin	Citrate	Tartrate	Succinate	Malonate	Oxalate	Lactate	L-Aspartic acid	Glycine	L-Lysine, 0.0062 M	L-Lysine, 0.0125 M
S-activator	I	A	220	200				104				
		B	232	159	180	127	146	99	108	91	120	
		C	170		144		100			90	100	
		E	255		186		156		156	112		50
	II	E	320		166		130		186	124		52
	Mean		239	180	167	127	144	102	150	104	110	51
Spontaneous activator	I	A	125	200								
		B	116	146	147	113	134	109	44	81	0	
		C	144		108		120			60	16	
		E	300		190		214		108	84		0
	II	E	295		240		180		86	58		0
	Mean		196	173	171	113	162	99	79	71	8	0
Bovine plasmin	I	C	106		96		112			84	52	
		E	112		114		108		108	92		64
	II	E	100		110		98		100	96		58
	Mean		106		107		106		104	91	52	61
Trypsin		A	120					108				
		B	127	116	127	107	104	102	100	106	105	
		C	124		94		64			96	56	
		D	100		88		88		80	100		116
		E	106		124		98		104	100		104
	Mean		115	116	108	107	89	105	95	101	81	110
Chymotrypsin		CDE	103×		99×		99×		95+	98+		103+
Aspergillus protease		CDE	100×		108×		90×		87+	120+		112+
B. subtilis protease		CDE	81×		95×		74×		93+	106+		110+

I and II represent different preparations of active agents. Final concentration of added compounds was 0.0125 M. Different batches of fibrinogen (ABCDE) were used to prepare the fibrin plates and one series of experiments (batch B: 2-3) was performed with each batch. For the 3 last proteases only means of values for 3 (×) and 2 (+) batches of fibrinogen are given (max. dev. 15%).

prepared in which the globulin concentration was one-half of the original concentration in serum. The following proteases were used: *Bovine plasmin.* A spontaneously active preparation of bovine globulin(6). *Trypsin.* A 0.05% solution of bovine crystalline trypsin (Armour, 50% $MgSO_4$) in N/400 HCl, diluted 1:25 with buffer before use(4). *Chymotrypsin.* A 0.1% solution of crystalline chymotrypsin (Armour, 50% $MgSO_4$), diluted 1:15 with buffer before use. *Aspergillus protease*(7) 0.02% in buffer. *B. subtilis protease*(7) 0.05% in buffer. *Barbital buffer.* 0.1 M; pH 7.6. Fibrinolytic activity was estimated by the fibrin plate method(4,8) and the active agents were used in concentrations which produced normal fibrinolytic activities of the same magnitude. Commercial samples of high purity were used of: sodium citrate, sodium succinate, sodium oxalate, sodium sulfate, sodium fluoride, sodium-potassium tartrate, lactic acid, malonic acid, L-aspartic acid, glycine, dihydrochlorides of L-lysine and L-ornithine, glycerol, glucose, lactose, ethanolamine, and pyridine. Neutralized solutions of these compounds were added to freshly prepared(8) fibrinogen solutions. Equal amounts of buffer were added and the mixtures were diluted with saline to a concentration of 0.1% fibrinogen, an ionic strength of 0.15(8), a pH between 7.55 and 7.60 (controlled by a glass electrode), and a concentration of added compounds of 0.0125 M. The final solutions were used to prepare fibrin plates. The activities found on plates with fibrin containing added compounds were con-

verted into percentages of the activity on plates containing normal fibrin by interpolation on dilution curves obtained with the appropriate active agents(4). The standard deviation on a single estimation is 11%(9). Mean values of duplicate estimations (each consisting of 3 replicates(4)) were used.

Results. The activity of the plasminogen activator preparations and plasmin was not significantly influenced by a calcium precipitant (sodium fluoride, 5 experiments), a divalent inorganic anion (sodium sulfate, 6 experiments), and some organic hydroxyl compounds (glucose, 5 experiments; lactose, 3 experiments, and glycerol, 3 experiments).

The effects of a number of organic acids are summarized in Table I. The enhancing and inhibitory effects were pronounced only in experiments using *activator preparations* and the effects seemed to depend on the number of carboxyl and amino groups. Lactate (amonocarboxylic acid) was without effect. All dicarboxylic acids produced a considerable increase in the activity of the activator preparations, and citrate (atricarboxylic acid) was most effective. However, the spontaneous activator produced more variable results than the S-activator. The difference between the 2 activator preparations was especially pronounced in the experiments with amino acids. The activity of S-activator was enhanced by aspartic acid (amonoaminodicarboxylic acid) and unaffected by glycine (amonoaminomonocarboxylic acid) while the activity of the spontaneous activator was somewhat decreased in both instances. Lysine (adiaminomonocarboxylic acid), in 0.0062 and 0.0125 molar, concentrations, produced respectively a strong and a complete inhibition of the spontaneous activator, while the S-activator was only moderately inhibited by the stronger concentration. Identical results were observed in one series of experiments with L-ornithine (0.0062 M). Some amines (ethanolamine and pyridine) did not significantly influence the active agents.

The activities of the *proteases* were generally not significantly influenced. Bovine plasmin and *B. subtilis* protease were moderately inhibited by lysine and di- and tricarboxylic acids respectively. The results of trypsin experiments on different batches of fibrin varied much more than those obtained in similar experiments with other proteases. A slight increase in the activity of trypsin was produced by citrate.

The difference observed in experiments with active agents from human blood and with bovine plasmin strongly supports the assumption that these substances are different entities, *i.e.,* plasminogen activators and plasmin proper(2,3). Only the activity of plasminogen activators was enhanced by carboxylic acids. The large variations in the activity of trypsin might be caused by a varying susceptibility of the different batches of fibrin to the direct fibrinolytic and to the plasminogen activating potency of this enzyme(7,10). The reaction between bovine plasmin and the fibrin substrate was unaffected by carboxylic acids. These results suggest that the increased fibrinolysis induced by carboxylic acids depends on the activation of the plasminogen in the fibrin substrate.

The final (enhancing or inhibitory) effect appeared to depend on the number of carboxylic and amino groups in the organic acid. Ginsburg, de Vries and Katchalski found that lysis of human plasma clots (induced by addition of streptokinase, chloroform or menstrual blood) was inhibited by polylysine, polyornithine and polyarginine. The monomers were ineffective under their experimental conditions(11). Polyaspartic acid obviated the antifibrinolytic effect of these basic amino-acid polymers *in vitro*(11) and prevented their toxic manifestations *in vivo*(12).

Summary. 1. Fibrinolysis produced by activation of plasminogen in bovine fibrin by plasminogen activator preparations from human blood was increased considerably by di- and tri-carboxylic acids and decreased by diaminomonocarboxylic acids. 2. Fibrinolytic activity of a number of proteases was generally not significantly influenced. Bovine plasmin was moderately inhibited by lysine.

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Blocking Effect of Ethyl Chloride Spray on Cardiac Pain Induced by Ergonovine.* (20871)

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Under suitable conditions ethyl chloride spray has proved effective in blocking the somatic component of cardiac pain when this occurs in acute myocardial infarction, angina pectoris and the shoulder-hand syndrome associated with coronary insufficiency(1-5). In initial trials by one of the authors (I.S.) ethyl chloride spray also relieved the angina induced by ergonovine in patients with effort angina and a normal electrocardiogram (Stein ergonovine test(6-8)). In such subjects with pre-existing coronary insufficiency, the intravenous injection of ergonovine induces electrocardiographic changes and/or pain which matches in character and location that induced by exertion; nitroglycerin promptly stops the pain and reverses the changes in the S-T segment and T-wave. The purpose of this investigation was to study more systematically the blocking effect of ethyl chloride spray in such patients with impaired coronary circulation and a positive Stein test.

Materials and methods. Thirty-six tests were done on 7 patients (44 to 70 years, 5 males and 2 females) who had a history of angina of effort and a normal resting 12-lead electrocardiogram. Three patients had moderate hypertension. One had a history of old myocardial infarction. None had congestive failure. At each test, the exact location of pain induced by walking was noted. A control blood pressure, pulse rate and resting electrocardiogram were taken. Leads I, II, III, aVr and V_4 were recorded on a direct-writing 2-channel Viso-cardiette. While Lead V_4 alone or Leads II and V_4 were being recorded, a colorless solution containing U.S.P. ergonovine maleate, 0.2 mg per ml of distilled water, was injected intravenously at the rate of 1 ml per minute. Injection was stopped when either pain or electrocardiographic changes appeared. Leads I, II, III, aVr and V_4 were repeated and blood pressure and pulse rate again determined about 1, 5 and 10 minutes after the end of the injection. When the test became positive, a sublingual dose of nitroglycerin (0.6 mg) was administered to terminate pain and/or electrocardiographic changes. This was usually given 6 to 8 minutes after the ergonovine, but occasionally only 2 minutes afterward if chest pain was intense. Electrocardiograms were taken until record became normal, usually 2 to 5 minutes after the nitroglycerin. At the first trial no more than 0.2 mg of ergonovine was injected. If 0.2 mg did not produce a positive effect, the

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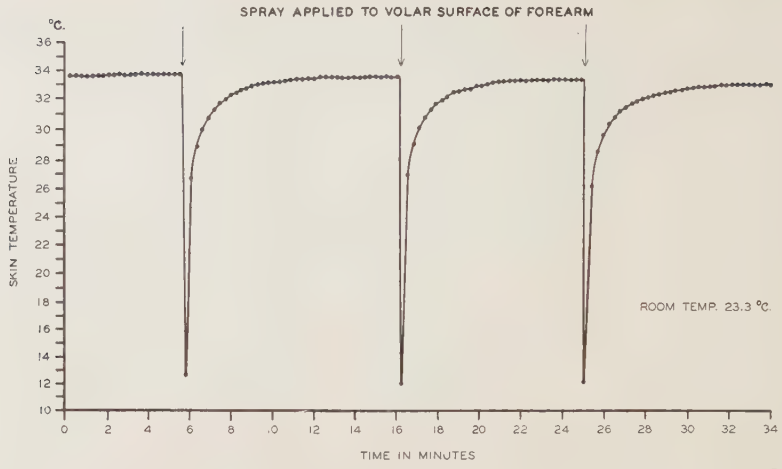

FIG. 1. Effect of 3 sweeps of ethyl chloride on temperature of skin surface as recorded by an iron-constantan thermocouple (Leeds and Northrup Speedomax).

patient was tested again with 0.4 mg one week later. No patient needed more than 0.4 mg. Thus, the threshold dose of ergonovine producing a positive test was determined for each patient. Criteria for positivity of the ergonovine test(6) are appearance of pain and/or S-T depression which amounts to at least 0.75 mm in limb leads and 1.5 mm in chest leads, or inversion of T-wave. Precautions in carrying out this test have been outlined(7). A control injection of physiologic saline was given each patient at some subsequent visit. Procedure was the same as for ergonovine and the same volume of solution used. In another series of tests on the 7 patients, 0.4 or 0.6 mg of nitroglycerin was given sublingually just before injection of ergonovine. In additional tests, ethyl chloride was sprayed over those areas of the chest, shoulders, arms or neck where pain had been produced by the ergonovine on an earlier occasion. The spray was applied in one series just *before* the dose of ergonovine was injected, and in another series when pain had appeared *following* ergonovine injection. Technic of applying ethyl chloride spray for relief of deep pain has been described (9,10). The jet stream is swept slowly over pain area in one direction; timing is such that one sweep lasts about 5 seconds with a 5 second break between sweeps. Since successive sweeps are applied to adjacent skin areas, any particular region is usually covered by the spray only once or perhaps twice. This manner of applying ethyl chloride spray may be visualized by inspection of the temperature curve from the skin surface during a single sweep (Fig. 1). The skin is not frosted. Perception of pinprick and light touch is retained in the sprayed area when tested immediately after completing such a single sweep.

Results. Results are shown in Table I. Pain and significant electrocardiographic changes were produced in all 7 patients by intravenous injection of ergonovine maleate. In Case 6, duplicate tests about 2 weeks apart yielded similar results (Table II). After intravenous injection of physiologic saline, no patient developed either pain or an abnormal electrocardiogram.

Nitroglycerin, 0.4 mg or 0.6 mg, given sublingually just before ergonovine, prevented pain in 5 and electrocardiographic changes in 6 of the 7 patients.

When the effective dose of ergonovine was administered and predicted pain appeared, ethyl chloride spray was applied to the pain areas. In 4 of 6 patients complete relief of pain occurred in from 30 to 60 seconds after start of spraying; 2 patients had partial relief (diminished intensity).

Ethyl chloride spray, when applied to appropriate skin areas just *before* ergonovine, prevented pain in 4 of 7 patients, delayed the onset of pain in 3 patients, but did not prevent the electrocardiographic changes in any (Table I). An illustrative test with prior

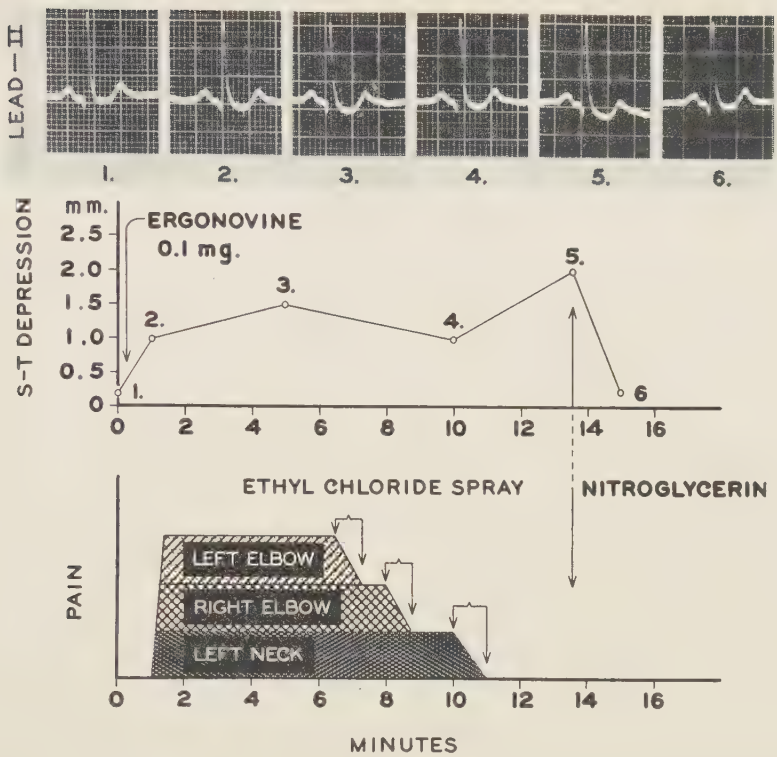

FIG. 2. Effect of ethyl chloride spray on ergonovine angina in B.G., a 49-year-old male. Note that as spray was applied to each painful area in turn, it stopped pain in that region within a minute. S-T segment remained depressed by 1 to 2 mm until nitroglycerin was administered 13½ min. after ergonovine injection. (In Fig. 2 and 3, time on the abscissa starts at *end* of ergonovine injection which occupies about 1 min.; control electrocardiogram was taken shortly before *start* of injection.)

application of ethyl chloride spray is shown in Fig. 3. In another patient, duplicate tests with prior spray showed essentially the same delay in onset of pain as compared with two control tests (Table II).

Discussion. The title of this paper implies that pain induced by ergonovine represents cardiac pain. This conclusion is based

TABLE I. Effect of Ethyl Chloride Spray on Pain and Electrocardiographic Changes Induced by Ergonovine in 7 Patients with Effort Angina and a Normal Resting Electrocardiogram.

Ergonovine dose, mg	Ergonovine		Saline		Nitroglycerin-ergonovine		Spray-ergonovine		Ergonovine-spray	
	Pain	ECG changes	Pain	ECG changes	Pain	ECG changes	Pain	ECG changes	Pain relief	Persistent ECG changes
.1	Yes	Yes	None	None	Yes	Yes	None	Yes	P*	Yes
.2	"	"	"	"	"	None	"	"	C	"
.4	"	"	"	"	None	"	"	"	C	"
.2	"	"	"	"	"	"	"	"		
.2	"	"	"	"	"	"	Yes	"	C	"
.1	"	"	"	"	"	"	"	"	C	"
.1	"	"					"	"		
.2	"	"	"	"	"	"	"	"	P	"

* P = partial; C = complete.

TABLE II. Duplicate Ergonovine Tests with and without Ethyl Chloride Spray (Patient 6).

Test No.	Date	Time to pain after ergonovine, min.	S-T depression (Lead II), max change, mm
		Ergonovine alone	
1	6/ 1/53	2	3.
3	6/13	1	2.
		Ergonovine preceded by ethyl chloride spray	
2	6/ 6	6	2.
4	6/22	4	3.

on a number of observations: The angina of ergonovine has a marked resemblance clinically to angina of effort in character, intensity and location of pain and also in such concomitants as sweating, pallor and extrasystoles or coupling. Similar electrocardiographic changes attend both effort and drug induced attacks. Nitroglycerin, which is known to dilate the coronary arteries, promptly stops pain and reverses the electrocardiographic changes after both exercise and ergonovine; it may also prevent these acute effects of ergonovine. It is reasonable to infer that angina of ergonovine depends on a coronary vasoconstrictor mechanism, especially since ergonovine fails to cause a significant rise in blood pressure or acceleration of heart rate as an accompaniment of pain.

That ethyl chloride spray blocks pain induced by ergonovine is in line with numerous observations concerning relief of pathologic cardiac pain by procedures altering the flow of nerve impulses from the somatic reference zone(1-4,11-13). So far as we know, however, the only other studies which show that standardized visceral pain may be *prevented* by treatment of somatic reference zone are those of Theobald(13) and Lindgren(12).

In our study, dissociation of cardiac pain and electrocardiographic changes characteristic of coronary artery disease is clearly shown in the combined effects of ergonovine and ethyl chloride spray. Russek *et al.*(14) also note such a dissociation at times in their standard exercise test. Another example of the divergence of pain and electrocardiographic changes is the clinically silent, acute myocardial infarct.

What is the mechanism by which ethyl chloride spray relieves cardiac pain when applied to the somatic reference zone? Promptness of relief afforded by the spray after ergonovine and after acute myocardial infarction(3) suggests interruption of a reversible neurogenic process responsible for maintaining the pain cycle independent of initiating cause. It seems likely that the mechanism is mainly that of counterirritation. Thus, the initial cold shock sets up an intense barrage of afferent impulses, followed by post-stimulatory depression and alteration of the central excitatory state. This is in line with Gammon and Starr's(15) finding that if counterirritant drugs and physical agents such as cold are to relieve pain effectively, the degree of initial stimulation must be of high intensity and close to that which produces pain. The intermittent application of cold (ice) was the most effective of the agents tested. Another factor in the relief of pain by ethyl chloride spray may be a brief hypesthesia due to cold, momentarily reducing the stream of afferent cutaneous impulses, in phase with subnormal (post-stimulatory) state of conduction in the central nervous system. It is further postulated that such a brief interruption of the neurophysiologic process responsible for pain can lead to permanent relief.

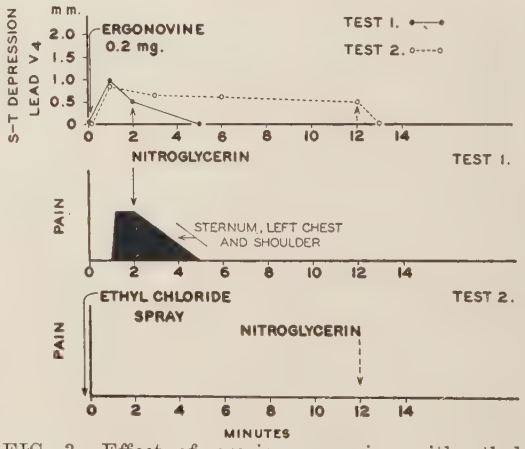

FIG. 3. Effect of previous spraying with ethyl chloride (Test 2) on pain and electrocardiographic changes induced by ergonovine (Test 1) in A.F., a 61-year-old female. Note: (1) S-T depression was similar in both tests; (2) after spraying sternum, left chest and shoulder, pain did not appear, and (3) electrocardiographic changes persisted in absence of pain for 12 min. (until nitroglycerin).

The effect of ethyl chloride spray probably can not be ascribed to reflex circulatory changes in the myofascial structures of the reference zone since in other experiments(16) we found that the spray relieves deep pain due to ischemic muscle work while the circulation to the region remains occluded. Gammon and Starr(15) observed that subcutaneous pain also was relieved by intermittent cold during occlusion of the circulation.

Summary. Thirty-six tests were carried out on 7 patients with effort angina but a normal resting electrocardiogram, in whom intravenous injection or ergonovine (0.1 to 0.4 mg) induced angina and electrocardiographic changes. Nitroglycerin, 0.4 or 0.6 mg given sublingually just before ergonovine, prevented pain and electrocardiographic changes in most patients. When angina had been induced by ergonovine and ethyl chloride spray was applied to pain areas of chest, shoulders, arms or neck (reference zone of cardiac pain in each patient), pain was uniformly relieved. Relief of ergonovine angina by ethyl chloride spray required less time than by nitroglycerin. When ethyl chloride spray was applied to pain areas just *before* ergonovine was injected, pain failed to occur in the majority, and the onset of pain was delayed in the remaining patients. In combination with ethyl chloride spray, ergonovine caused depression of the S-T segment even in the absence of pain. These results afford additional evidence of the previously reported effectiveness of ethyl chloride spray for relief of the somatic component of cardiac pain.

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Presence of Antidiuretic Material in Blood of Hypophysectomized Rats. (20872)

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It has generally been accepted that removal of the posterior pituitary results in disappearance of antidiuretic hormone. Several investigators, using different assay technics (1-3), have reported the absence of antidiuretic material from the blood of hypophysectomized rats.

In the course of studies of turn-over of water and metabolism of posterior pituitary hormone of hypophysectomized rats which were a month or more postoperative, it was observed that an appreciable number of the animals had antidiuretic material in their blood. This was not found in the blood of

recently hypophysectomized animals. This report describes these findings and other evidence which supports the concept that this antidiuretic material may be formed and released by the hypothalamus.

Methods and materials. Antidiuretic material was assayed by the method of Jeffers (4) which employs the alcohol anesthetized and hydrated rat and the intravenous injection of the material being tested. Details of the technic were as published by Ames and van Dyke(2), except that it was found that a diuresis could be more dependably produced when the interval between the administration of the anesthetizing dose of alcohol and the hydrating dose of water was increased to approximately an hour. In some animals hydration was maintained by the administration of small amounts of water and alcohol through a plastic stomach tube left in place throughout the assay. Hypophysectomized Sprague Dawley males were purchased from the Hormone Assay Laboratories. Careful dissection of the region of the sella turcica or histological sections of this region have not revealed detectable remnants of hypophysis. The marked atrophy of the endocrine system characteristic of hypophysectomized animals occurred in each case. A number of animals were demonstrated to have the inability to excrete water which is found in the hypophysectomized animal. The water test was not carried out in every animal because of the high mortality from water intoxication. In the initial experiment the animals were fed Purina Dog Chow, milk and vegetables *ad libitum*. All of these animals developed the marked cachexia of the hypophysectomized rat. Subsequently, other animals were fed a high protein, high calorie diet in which the principal source of protein was ground horsemeat. These animals gained weight although their endocrine organs all showed the usual posthypophysectomy atrophy. The two diets produced no differences in the amount of antidiuretic substance in the blood. Blood from hypophysectomized animals was obtained by heart puncture, under pentobarbital anesthesia. The assay was usually carried out on serum although the plasma levels were essentially the same. The blood was immediately placed in iced tubes and kept iced during separation of the serum. The serum was injected into the assay animal within 10 minutes after the blood was drawn. The hypothalami were obtained by taking a block of tissue about 5 mm wide from the floor of the third ventricle, extending from anterior of the optic chiasm to posterior of the mammillary bodies. This whole block of tissue was homogenized and diluted with 25 cc of physiological saline solution. Two-tenths cc of this solution was given intravenously. A comparable amount of cerebral cortex from one of the lateral lobes was used as a control. This tissue was never found to have antidiuretic activity.

TABLE I. Antidiuretic Activity of Serum of Hypophysectomized Rats.

Postoperative interval	No. of animals	ADS of serum, γu of pitressin per cc
$<$ 7 days	6	$<$ 30
$>$30 "	29	60–400

Results. The antidiuretic content of the blood of the hypophysectomized animals is shown in Table I. The blood of 29 hypophysectomized animals which were at least one month postoperative was tested and the blood of 21 was found to contain antidiuretic activity. Detectable antidiuretic activity was found in .05 to .3 cc of serum. An amount of antidiuretic substance equivalent to 20 microunits of pitressin was usually encountered in 0.1-0.2 cc of serum *i.e.* 60 to 400 microunits of pitressin per cc of serum. This is essentially the same amount as has been found in the blood of intact animals. Six recently hypophysectomized animals were tested. No activity was found in serum in amounts up to .5 cc.

The hypothalami of 7 animals hypophysectomized for more than 30 days were tested and found to contain activity equivalent to more than 5 milliunits of pitressin. The antidiuretic activity of the hypothalami of 7 recently hypophysectomized animals was considerably less than 5 milliunits, but the exact amount of antidiuretic activity has not yet been determined.

Discussion. The presence of antidiuretic

activity in the blood of hypophysectomized animals suggests either that the posterior pituitary has not been removed or that the structures remaining have reorganized to permit release of antidiuretic material.

Although Bodian and Maren(5) have reported the persistence of neurohypophyseal tissue in hypophysectomized rats, any remnants in the animals used in this study must have been extremely small. The absence of antidiuretic activity in blood soon after hypophysectomy and its appearance somewhat later suggest that a functional reorganization has taken place permitting formation and release of antidiuretic hormone.

There is considerable evidence to support the concept of neurosecretion by the hypothalamus. This hypothesis holds that antidiuretic hormone is formed in the nuclei of the hypothalamus and that it progresses along nerve cells to the posterior lobe from which it is released(6). The data presented here tend to support the concept that the hypothalamus forms antidiuretic hormone and in addition, they suggest that under appropriate circumstances, it releases antidiuretic hormone.

The finding of larger amounts of antidiuretic hormone in the hypothalami of the 30-day postoperative animals suggests that a reorganization has taken place permitting the hypothalamus to accumulate antidiuretic hormone. These data confirm the finding by Sato(7) in the dog of an increased amount of antidiuretic hormone after hypophysectomy. As pointed out by Melville and Hare(8), who found a decline of antidiuretic activity in the supraoptic region after hypophysectomy, the median eminence was included in Sato's extract. It was also included in our extracts. This area has been shown to contain pituicytes which produce vasopressin(9) and these cells are said to hypertrophy after hypophysectomy (10). It is probable that the increase in antidiuretic content of the hypothalamus occurs as a result of increases in activity of cells in the region of the median eminence and that the content of antidiuretic hormone in the supraoptic nuclei is actually decreased.

The ability of the hypothalamus of the hypophysectomized animal to secrete antidiuretic hormone is suggested by the demonstration by Mirsky *et al.*(11) of an increase in antidiuretic activity in the blood of hypophysectomized animals following noxious stimuli. Anatomical evidence suggesting this possibility has been found by Laqueur(12) who has demonstrated structural reorganization in the region of the median eminence of rats after hypophysectomy suggesting the possibility that this tissue may be able to secrete. This concept would help to explain the demonstration of antidiuretic substances in the blood of some humans with diabetes insipidus(13). Although the antidiuretic activity of the blood of these patients tended to be lower than normal, there was appreciable activity detectable in several patients who had severe diabetes insipidus. It was suggested that in these cases the diabetes insipidus might be due not only to a decrease of an antidiuretic factor, but also to an increase in diuretic substances.

Conclusion. 1. The blood of rats hypophysectomized for a month or more contains antidiuretic activity in amounts similar to those found in normal animals. 2. In the blood of animals hypophysectomized for less than one week there is no detectable antidiuretic material. 3. The hypothalami of recently hypophysectomized animals contain less antidiuretic activity than the hypothalami of animals hypophysectomized for a longer time.

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Variations in Sensitivity to Anaphylaxis and to Histamine in Inbred Strains of Mice.* (20873)

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(Introduced by Richard Thompson.)
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Following a study demonstrating that there was genetic variation in the ability of mice to form circulating antibody(1), experiments were designed to determine whether or not similar variation could be demonstrated for the ability to react with anaphylactic shock; and to determine the extent that anaphylaxis was dependent on circulating antibody. This further led to an inquiry concerning the basic mechanism of anaphylaxis in the mouse, especially to a re-examination of the possibility of a relationship between anaphylaxis and histamine sensitivity.

Materials and methods. Anaphylaxis. Every attempt was made to operate under conditions identical to those prevailing in the study on circulating antibody(1). The same 5 inbred strains of mice—DBA/1 Jax, BALB/C Jax, C3H/Jax, C57BL/6 Jax, and A/He Jax, all sufficiently inbred to insure genetic uniformity in succeeding generations, were used. They were of mixed sexes, and over 4 months old. The amount of alum precipitated egg white, the intra-abdominal route of injection, and the time interval did not vary between the 2 experiments.

Mice were given a challenging intravenous injection of 15 mg of crystalline egg albumin in 0.25 ml of 0.85% NaCl, an amount estimated to furnish an antigen excess. The mice were carefully observed for the characteristic signs of anaphylaxis, and the findings were recorded in accordance with the following: convulsions with or without death, *positive,* severe; muscular spasms or paralysis without death, *positive,* mild; no effect or very slight agitation, in absence of above, *negative.* The time of death was recorded for each animal

TABLE I. Effect of Circulating Antibody on Sensitivity to Anaphylaxis.

	DBA	BALB/C	C3H	C57BL/6	A/He
No. with circulating antibody*	28	23	22	26	23
No. injected	28	27	22	27	24
Rank*†	Excellent-good	Excellent	Good	Excellent	Fair
Anaphylaxis:					
Severe with death	4	12	4	5	24
" without death	0	3	0	0	8
Mild shock	0	0	1	1	0
Negative	28	10	26	36	1
Total	32	25	31	42	33

* Results from (1).
† Based on a statistical analysis considering both number positive and titer.

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TABLE II. Comparison of Sensitivity to Histamine and to Anaphylaxis.

	DBA	BALB/C	C3H	C57BL/6	A/He
No. with anaphylactic shock*† / No. injected	15/32 (47%)	23/27 (85%)	7/31 (23%)	15/42 (36%)	32/33 (97%)
Median reaction time—min.	25 (6-360)	85 (11-960)	87 (46-150)	26 (13-120)	23 (15-47)
No. reacting to histamine† / No. injected	15/16 (93%)	14/16 (88%)	1/16 (6%)	12/16 (75%)	15/16 (94%)

* Survivors of first shocking inj. were tested 7 days later. These figures are total No. reacting in the two test periods.

† No statistical difference due to sex was ever apparent.

dying of the shock.

For every strain, 5 to 10 normal control animals were injected intravenously with the antigen. In no case did an animal show any effect from this injection. Results of this anaphylaxis experiment are recorded in Table I, where they are compared with the amount of circulating antibody.

One week after the first shocking injection, survivors were injected intravenously with the same amount of antigen. The total number of animals reacting in both of the two trials in each strain is recorded in Table II.

Histamine. An Eimer and Amend preparation of crystalline histamine diphosphate was used. Ten per cent solutions were made in 0.85% NaCl daily, immediately prior to use. In preliminary tests, it was found that by the intravenous route a minimum of 0.5 mg per gram of body weight was necessary to elicit a reaction. This standard dose was then employed in determining the sensitivity of the different strains.

Two-months-old mice of the same strains were employed. This age was selected to make the results comparable to other work on histamine sensitivity in the mouse(2). Eight males and 8 females of each strain were tested, except in the A/He strain, in which 11 males and 5 females were used. A positive reaction was recorded when the animal suffered a frank convulsion, with or without death. No mild reactions were observed.

Results. Table I shows, for each of the 5 mice strains, the percentage of animals having circulating antibody, and the incidence of anaphylactic shock after the same time interval, and under the same conditions of immunization. The two sets of data were rank-ordered in terms of the amount of circulating antibody present and the severity of anaphylactic shock, respectively, and were compared by using Spearman's rank-difference correlation method. A Rho of –0.8 was obtained. A rough test of the significance of correlation was obtained by comparing it with its standard error of 0.16. This comparison showed the correlation to be significant below the 1% level of confidence. This statistical analysis indicates an inverse relationship between ability to shock anaphylactically and the circulating antibody titer. On examination of the data for individual strains, however, it is seen that this relationship does not hold for every strain.

Table II contains data showing the extent of sensitivity to histamine and to anaphylaxis for each of the 5 mouse strains. The extent of inter-strain variability was determined by the method of Chi square. Each strain was compared with every other strain in both cases.

The results expressed in Table II indicate the following: a) a genetic influence on the ability to shock; and b) a strain variation in the sensitivity to histamine. With respect to the former, strains A/He and BALB/C were not significantly different from each other, but were different from the other 3 strains; strains DBA and C57BL/6 were not different from each other, but were different from A/He and BALB/C; strain C3H was significantly different from DBA, but not from C57BL/6.

A strain difference in sensitivity to the intravenous injection of histamine is also apparent, with the C3H strain significantly different from all the others, being the most resistant.

When the results of the two determinations are compared, it is seen that the C3H strain, the most resistant to histamine, was among the three strains most resistant to anaphylaxis; and the A/He strain, one of the two strains most susceptible to anaphylaxis, was one of the strains most susceptible to histamine. Beyond this, the prediction of a relationship between histamine and anaphylactic sensitivity will not hold.

Discussion. A genetic variation in the ability of mice sensitized to egg albumin, to respond to a subsequent intravenous injection of this antigen by anaphylactic shock, is apparent from the results presented in Table II.

No direct relationship exists between the circulating antibody titer and the sensitivity to anaphylaxis. On statistical analysis the possibility of an inverse correlation is indicated, but it remains for future work to determine the significance of this finding. Further suggestions that these two phenomena are not related in the mouse are found in the work of Weiser *et al.*(3) who found that anaphylactic sensitivity persisted long after precipitins were no longer demonstrable; and by Malkiel *et al.*(4) who were unable to demonstrate precipitable or non-precipitable antibody, or passively transferable antibody, in the circulation of actively sensitized mice. Active anaphylaxis has also been used to demonstrate the antigenicity of homologous malignant tissue in the mouse(5), again in the absence of demonstrable circulating antibody. These findings, together with the observation herein reported that circulating antibody and anaphylaxis are not positively correlated, support the thesis that the antibody responsible for mouse anaphylaxis does not reside in the circulation.

The mechanism of anaphylaxis in the mouse, although it has received some investigation, remains obscure. The repeated findings(2,6,7) that enormous doses of histamine are necessary to produce shock in the mouse, plus the suggestion that the entire body of the mouse is incapable of yielding sufficient histamine to produce the reaction(6) is evidence that the shock syndrome is not mediated through the action of histamine. It may not be coincidental that the results reported in Table II show that the C3H strain of mice, the most resistant to anaphylactic shock, is also the most resistant to histamine. On the other hand, the A/He strain, the most susceptible to anaphylaxis, is no more sensitive to histamine than the other strains studied. Further work on pertinent differences between these two strains is in progress.

In contrast to the intra-abdominal route of injection of histamine, as employed by many workers, the intravenous injection as used here requires a smaller quantity of the drug to produce a reaction. The finding that there is no statistical difference in the reaction of the two sexes to histamine is in disagreement with previously reported observations(2). This may or may not be due to the different route of injection.

Summary. 1. Based on the reactions of mice of 5 inbred strains sensitized to egg albumin, evidence is presented which indicates a genetic variation in the ability of mice to respond with anaphylactic shock. 2. The response by anaphylaxis was not found to be positively correlated with the amount of circulating antibody, and it is suggested that anaphylaxis in the mouse is not dependent on this antibody. 3. One inbred strain of mouse, C3H, which was found to be significantly more resistant to anaphylaxis, was also more resistant to histamine. A second strain, however, very susceptible to anaphylaxis, was no more sensitive to histamine than any other. 4. No sex difference in sensitivity to histamine or to anaphylaxis was demonstrable.

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Effect of Cortisone Acetate on Aspartic-Glutamic Transaminase Activity of Mouse Tissues.* (20874)

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The aspartic-glutamic transaminase activity of the liver, kidney and muscle of the rat is reported(1) to be unchanged after adrenalectomy and replacement doses of cortisone. On the other hand, arginase activity of the liver and kidney is reduced after adrenalectomy(2-4) and restored to and above normal by cortisone(2,3). The stimulatory effect of cortisone on the arginase activity of the liver and kidney is also demonstrable in normal mice but only after about 2 days of treatment (5). Since arginase and transaminase are closely related in their role in the intermediary metabolism of protein, it seemed important to reinvestigate the transaminase activity of the liver and kidney under conditions in which cortisone produced a definite increase in arginase activity(5). Furthermore, since cortisone seems to influence the metabolism of the heart and this tissue contains the highest activity of transaminase(6) it also was studied.

Procedure. Mice of the dba-1, 20C57B1 line 6, and BBF strain (Jackson Laboratory)† were maintained at not more than 5 in a glass jar containing wood shavings. They were fed *ad libitum* Purina laboratory chow checkers or a prepared diet composed of casein 16.7, sucrose 61.2, hydrogenated vegetable oil (Primex) 7.4, dry Brewers' Yeast (Fleischmann's No. 2019) 9.2, Celluflour 1.8, Wesson's salt mixture 3.7 and, 3 times/week a supplement of 2 drops of cod liver oil and 1 drop of 40% tocopherol concentrate of vegetable oils diluted 10 fold with Wesson oil. No difference in results between strains of mice or due to the use of the two diets was detected. *Hormone.* The cortisone acetate‡ was prepared in cylindrical pellets weighing approximately 15 mg each and implanted subcutaneously. The average amount absorbed decreased as the duration of the experiments was increased, (Table I)(5). *Autopsy. Preparation of tissue.* Food, but not water, was removed from the mice 19 to 24 hours before autopsy. The mice were killed by spreading the vertebrae at the neck to sever the spinal cord and were immediately bled to death by cutting the blood vessels of the neck. The entire liver, kidneys, and heart were removed for the enzyme determination, except in cases in which a small section of the heart was used for histological preparations. The organs were weighed on Roller-Smith torsion balances, and immediately placed in ice-cold water, homogenized in an all-glass apparatus and diluted to volume. *Enzyme determination.* The activity of the aspartic-glutamic transaminase was determined by the procedure of Tonhazy *et al.*(7) but the values are expressed on the basis of fresh tissue weight.

Results. Body and organ weights. The body, spleen, thymus and liver weights decreased and the kidney weight increased (Table I) as previously reported(5). The heart also increased in weight. The younger mice, however, did not show these changes in heart and kidney weight (Table I).

Transaminase activity. The activity of the transaminase decreased in the liver both on a total and a u/g basis. (Table II). The decrease was evident only after 14 days when it was maximal. On the other hand, the activity of this enzyme in the heart and kidney increased. The above changes did not appear until between 2 and 7 days of treatment. Similar results were obtained in another series of experiments of 4 and 10 days duration§

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† Mice made available from funds provided by the Cooper Foundation to the Roscoe B. Jackson Memorial Laboratory.

‡ Cortisone acetate was provided by Merck and Co. through the courtesy of Dr. Elmer Alpert.

§ Adenosinetriphosphatase activity was also determined. Total units in liver decreased 25 and 41%, in kidney 14 and 13% and in heart no change for 4 and 10 day experiments respectively.

TABLE I. Effect of Cortisone Acetate on Body and Organ Weights of Mice.

Treatment, days	Cortisone Absorbed, mg/day	No. mice	Body wt, g Initial	Body wt, g Change	Spleen, mg	Thymus, mg	Liver, mg	Kidney, mg	Heart, mg
Control		24*	25.1	+2.5*	133 ± 9.3	40 ± .1	1230 ± 48	249 ± 6.6	99 ± 3.4
2	1.0	6	24.8		44 ± 2.5	9 ± .1	1230 ± 79	231 ± 17.6	93 ± 6.7
7	.5	6	23.8	—1.4	61 ± 12.6	5 ± 1.4	1110 ± 78	284 ± 12.0	116 ± 4.3
14	.4	6	23.8	—2.3	62 ± 5.7	0	1090 ± 47	295 ± 14.3	112 ± 3.5
21	.35	6	24.3	— .2	50 ± 3.8	0	1090 ± 43	338 ± 18.7	118 ± 10.0
Control		10†	15.3	+1.5‡			810 ± 25	208 ± 8.	81 ± 2.9
4	.5	10†	15.2	+ .1			720 ± 16	199 ± 9.	83 ± 1.9
10	.3	5†	14.5	—1.8			520 ± 40	217 ± 15.	81 ± 4.3

* Values of the 6 controls of each period averaged and presented with the stand. error of the mean, $\frac{\sqrt{\frac{\Sigma X^2}{N} - \bar{X}^2}}{\sqrt{N-1}}$. Body wt change is for 21 day group.

† 5, 8, and 2 females in respective groups.

‡ Represents change in 10 days.

(Table II). The higher control values obtained in this series are very likely due to the younger age of these animals. Both male and female mice of the dba-1 strain were used in the latter study but no difference due to sex was detected.

Discussion. It is of interest that the decrease in transaminase activity of the liver occurred under circumstances which had produced an increase in arginase activity(5). In both instances, the change in enzyme activities did not appear until after the initial protein catabolic phase had disappeared.

The change in the activity of the transaminase of the liver apparently is related to the excess cortisone administration. Neither adrenalectomy nor replacement therapy with cortisone affect the activity of this enzyme in the rat(1). If protein depletion is related with the changes in the activities of these two enzymes, it is peculiar to the action of cortisone. Loss of protein by dietary means does not result in changes in the activities of these enzymes(8).

In contrast to the liver, the kidney responds to an overdosage of cortisone by an increase in both the transaminase and arginase activities with a concomitant small increase in weight. Furthermore, the heart responds in a manner similar to the kidney.

Summary. Cortisone acetate produces in the mouse after 7 days a decrease in the aspartic-glutamic transaminase activity of the liver but an increase in that of the kidney and heart.

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TABLE II.*
Effect of Cortisone Acetate on Aspartic-Glutamic Transaminase Activity of Mouse Tissues.

Treatment, days	Liver Total u	Liver u/g	Kidney Total u	Kidney u/g	Heart Total u	Heart u/g
Control	118 ± 3.2	97 ± 3.0	15.0 ± 2.7	58 ± 1.9	13 ± 1.5	132 ± 4.7
2	116 ± 4.7	95 ± 4.6	13.0 ± 1.2	54 ± 1.5	11 ± 1.0	116 ± 3.9
7	115 ± 4.6	104 ± 12.8	23.3 ± 1.8	81 ± 3.3	18 ± .5	153 ± 5.6
14	79 ± 1.4	74 ± 3.3	18.0 ± .1	61 ± 2.4	17 ± 1.5	147 ± 9.9
21	84 ± 5.6	80 ± 5.2	23.0 ± 2.7	73 ± 4.1	22 ± 1.3	193 ± 10.4
Control	163 ± 5.4	202 ± 8.3	27 ± .8	128 ± 2.5	20 ± 1.0	246 ± 5.9
4	138 ± 5.4	192 ± 5.8	32 ± .9	159 ± 3.6	24 ± .7	288 ± 5.9
10	81 ± 6.8	157 ± 8.3	30 ± 4.0	144 ± 4.5	25 ± 1.7	308 ± 6.0

* See Table I and text for details.

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Prevention by Vitamin B_{12} of Protein Catabolic Action of Cortisone.* (20875)

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Large doses of vit. B_{12} partially counteract certain catabolic effects of cortisone in young rats fed diets deficient in this vitamin(1-3). Reductions in body, hair and thymus growth caused by cortisone were partially overcome by feeding 10 times the normal requirement for this vitamin. The beneficial effects were invariably accompanied by increases in food intake and by greater efficiency in converting food into body weight gains. It was suggested that (a) large doses of cortisone may increase requirements for vit. B_{12} and (b) administration of the vit. may enhance the availability of protein. The first was partially corroborated by Wahlstrom and Johnson(4), who reported that large doses of cortisone injected into baby pigs increased urinary excretion of vit. B_{12}. The results of the present experiments demonstrate that when young rats are fed *ad libitum*, vit. B_{12} can prevent an increase in urinary nitrogen losses produced by injecting large doses of cortisone.

Methods. Two similar experiments were performed in 90 rats, but since the results were essentially the same in both, only one will be reported. Fifty young male Carworth rats were divided into 5 uniform groups of 10 each, and fed the vit. B_{12}-deficient, corn-soybean diet previously described(1) for 20 days. After this depletion period, each group was treated as follows for 30 days: 1) no vit. B_{12}; 2) 200 μg vit. B_{12}‡/kilo of diet; 3) cortisone‡; 4) cortisone and 200 μg vit. B_{12}/kilo of diet; 5) same as 4 but pair-fed to group 3. Groups 3, 4 and 5 received one mg of cortisone acetate daily by subcutaneous injection for the first 10 days, 2 mg daily for the second 10 days, and 4 mg daily for the third 10 days. During the initial depletion period, three 24-hour urine specimens were collected at approximately weekly intervals and analyzed for total nitrogen by the micro-Kjeldahl procedure(5). After this, urinary nitrogen was determined on all rats of each group on the 5th and 10th days of each 10-day treatment with a particular dose of cortisone. Measurements of body weight and food consumption were made every 2 days. The rats were housed in metal cages with raised screen bottoms in an air-conditioned room at a temperature of 78 ± 1°F.

Results. The 50 rats averaged 58.3 g each in body weight at the beginning and 94.9 g each at the end of the 20-day depletion period. Body growth trends after this period are shown in Fig. 1. Rats which were continued on the vit. B_{12}-deficient diet (group 1) reached a final average body weight of 140.0 g as compared to 192.4 g each for the rats fed vit. B_{12} (group II). Growth of rats given cortisone but no vit. B_{12} (group III) was completely suppressed by one and 2 mg of hormone daily, and loss of body weight re-

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‡ Crystalline vit. B_{12} and cortisone acetate (Cortone) were furnished through the kindness of Dr. L. Michaud of Merck and Co., Rahway, N. J.

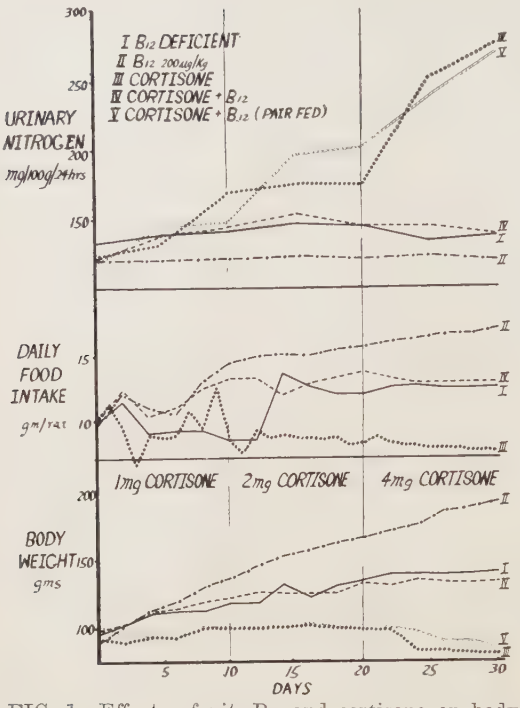

FIG. 1. Effects of vit. B_{12} and cortisone on body wt, food intake and urinary nitrogen of young male rats.

sulted when 4 mg were injected daily. When vit. B_{12} was added to the diet of cortisone-injected rats (group IV), growth inhibition was prevented although it did not reach the level of group II. Vit. B_{12} did not prevent growth inhibition by cortisone when food intake was limited (group V) to that of the rats given cortisone without vit. B_{12} (group III). Food intake and efficiency of utilization for body growth (Table I) were greatest in rats treated only with vit. B_{12} (group II) and least in rats given cortisone without vit. B_{12} (group III). Food intake was increased when vit. B_{12} was given to the cortisone-treated rats (group IV), but efficiency of food utilization was well below that of either group I or II.

The average daily excretion of urinary nitrogen per 100 g of body weight (Table I) during the vit. B_{12}-deficiency period apparently was not altered after the vitamin was added to the diet (group II). Although nitrogen values of group I are slightly higher than those of group II, these differences are not believed to be statistically significant. All levels of cortisone significantly increased urinary nitrogen losses in vit. B_{12}-deficient rats, and the largest dose of hormone doubled the initial nitrogen values (group III). The addition of vit. B_{12} to the diet largely prevented any increase in urinary nitrogen excretion by cortisone under *ad libitum* feeding conditions (group IV), but was completely ineffective when food intake was limited (group V) to that consumed daily by the rats of group III.

Discussion. These results demonstrate that in young rats fed *ad lib.*, vit. B_{12} can largely counteract the protein catabolic action of large doses of cortisone. This aids in explaining the ability of vit. B_{12} to overcome partially the cortisone-induced inhibition of body, hair and thymus growth(1-3). Vit. B_{12} apparently was unable to alter normal urinary nitrogen values, an observation in accord with others who have failed to demonstrate a specific effect of this vitamin on protein metabolism(6-9). These workers have suggested that vit. B_{12} may be involved primarily in utilization of carbohydrate and its transformation to fat, rather than in protein metabolism. The effectiveness of vit. B_{12} in preventing an increase in urinary nitrogen loss by cortisone may be explained by the observations of Engel(10), who noted that administration of glucose or amino acids to fasted nephrectomized rats prevented the usual increase in blood urea elicited by injecting adrenal cortical extracts. It is probable therefore, that vit. B_{12}, by increasing appetite and food intake, increases the availability of glucose or amino acids to the organism and thus overcomes the protein catabolic action of cortisone.

Vit. B_{12} failed to prevent cortisone from increasing urinary nitrogen losses when food intake was limited to that consumed by cortisone-treated rats on the vit. B_{12}-deficient diet. This is in agreement with Rupp and Paschkis(11), who similarly noted that vit. B_{12} was ineffective in counteracting protein catabolic effects of large doses of cortisone in rats limited in their food allowance. Apparently vit. B_{12} does not exert any growth effect in young rats when food intake is reduced to that of rats on a vit. B_{12}-deficient diet(12,13).

TABLE I. Effects of Vit. B_{12} and Cortisone on Urinary Nitrogen and Food Intake.

Treatment	Avg food intake, g Total	Per g gain body wt	Avg mg N/100 g/24 hr Before treatment	Treatment period in days 5	10	5	10	5	10
I. No V‡	327.6	7.47	132.56* ± 5.52†	139.51 ± 4.68	141.92 ± 4.87	146.87 ±12.5	144.01 ± 2.02	133.87 ± 9.70	136.16 ± 8.50
II. V	386.0	3.71	120.01* ± 5.45	120.71 ± 2.50	121.28 ± 2.94	123.58 ± 2.75	120.96 ± 8.46	122.72 ± 2.34	119.23 ± 1.20
				1 mg cortisone		2 mg cortisone		4 mg cortisone	
III. C, no V	251.9	—	123.34* ± 4.39	132.68 ±19.43	169.42 ±30.71	175.69 ±37.38	169.85 ±22.19	251.43 ± 4.65	276.78 ± 2.64
IV. C and V	357.0	9.92	120.64* ±10.77	137.64 ± 7.17	144.44 ± 6.14	154.09 ± 6.51	144.34 ± 9.15	144.81 ± 2.03	137.63 ±16.8
V. C and V (pair fed to group III)	251.9	—	118.14* ± 8.00	145.34 ± .18	148.46 ± .10	192.24 ±11.45	201.59 ± 6.00	238.74 ±10.5	270.70 ± .47

* Avg of 3 figures. † Stand. error of mean. ‡ V = Vit. B_{12}; C = cortisone.

These observations, however, cannot be interpreted as indicating that a mere increase in food consumption in the absence of adequate intake of vit. B_{12} would suffice to overcome the protein catabolic action of large doses of cortisone.

It will be noted (Fig. 1) that although vit. B_{12} prevented growth inhibition by cortisone (group IV), it did not increase growth to the level of the rats given vit. B_{12} without cortisone (group II). This was true even though the rats were fed approximately 10 times their normal requirement for this vitamin. In explanation, it can be seen (Table I) that despite the return to practically normal urinary nitrogen and food intake levels, the efficiency of food utilization for body weight gains was only about 1/3 as great in the former as in the latter group. Thus, the doses of cortisone employed antagonized one of the normal actions of vit. B_{12} and induced a considerable wastage of food. This emphasizes that there are limits to the ability of vit. B_{12} to overcome the inhibitory effects of cortisone on growth processes, and that the hormone may increase requirements for other factors than vit. B_{12}. In this connection, previous work has shown that a combination of vit. B_{12} and aureomycin is more effective in counteracting the inhibitory actions of large doses of cortisone in young rats than vit. B_{12} alone(1-3).

Summary. 1. Fifty young male rats were divided into 5 uniform groups and were fed a vit. B_{12}-deficient diet for 20 days. For the following 30 days, one group received 200 μg of vit. B_{12}/kilo of diet, another was continued on the deficient diet, and the other 3 groups were injected with large doses of cortisone acetate with or without vit. B_{12}. 2. Vit. B_{12} alone did not alter daily urinary nitrogen excretion/100 g body weight, but increased appetite and body weight gains. Without vit. B_{12}, cortisone significantly increased daily urinary nitrogen losses and reduced body growth and food consumption. Vit. B_{12} largely prevented these protein catabolic actions of cortisone under *ad lib.* feeding, but was ineffective when food intake was reduced to that of rats given cortisone without vit. B_{12}. 3. It is concluded that (a) vit. B_{12} can prevent increased urinary nitrogen losses induced by cortisone, probably by increasing appetite and thereby enhancing the availability of carbohydrate or protein to the organism (b) large doses of cortisone can markedly inhibit the ability of vit. B_{12} to transform food into body weight gains. Other implications of these findings are discussed.

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Intracerebral Adaptation to Mice of the Leon Strain of Type 3 Poliomyelitis Virus.* (20876)

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The adaptation to mice of the Leon Strain of type 3 poliomyelitis virus employing the intraspinal route has been reported by Li and Habel(1). Their results have been confirmed by Casals, Olitsky, and Brown(2). In a more recent paper Li and Schaeffer(3) have described a further modification of the mouse adapted line of the Leon Strain; continued mouse passage increased the virulence for mice but decreased intracerebral infectivity for the monkey. Both Li and Schaeffer(3) and Habel (ref. cited in 3) noted that this strain would occasionally infect mice by the intracerebral route. The present report gives details of procedures whereby this virus has been adapted to mice resulting in a line that is pathogenic via the intacerebral route as well as the intraspinal.

Methods and materials. Technic of criss-cross passage. In the adaptation experiments to be described criss-cross passages were used between tissue cultures and mice, injecting tissue culture fluid intracerebrally in the mouse and recovering, in tissue cultures, the virus from the cords of animals thus paralysed. The 2nd tissue culture passage was again inoculated intracerebrally and the criss-cross passages repeated in the same way. The tissue culture fluids of various passages were then titrated in mice by the intracerebral and intraspinal routes. *Viruses.* Drs. Li and Schaeffer, to whom we express our thanks, were kind enough to send to us their line of the Leon Strain, adapted for intraspinal infection of mice. The material consisted of 10% cord suspension and was taken from the 81st mouse passage. During our experiments, this strain was typed frequently in tissue cultures and identified as type 3 poliomyelitis virus. A second strain (Lansing, type 2), which is well adapted for the intracerebral route in mice, was used for comparative titration both intraspinally and intracerebrally in mice. *Test in mice.* CFW mice (15-20 g) were used throughout these experiments and a standard inoculum of 0.025 ml was employed for both the intracerebral and intraspinal route. The technic of the intraspinal inoculation was the same as described by Habel and Li(4). Average loss of mice paralysed by trauma was about 10% in the present studies. Animals found dead or paralysed after 12 to 24 hours were eliminated from the final results. Only cords of animals found paralysed after cerebral inoculation were pooled and used as 10% tissue suspension for inoculation into tissue cultures. It was felt that the ability to invade the cord might be one characteristic of a pathogenic virus. The cord suspension was prepared in distilled water and clarified with streptomycin according to a

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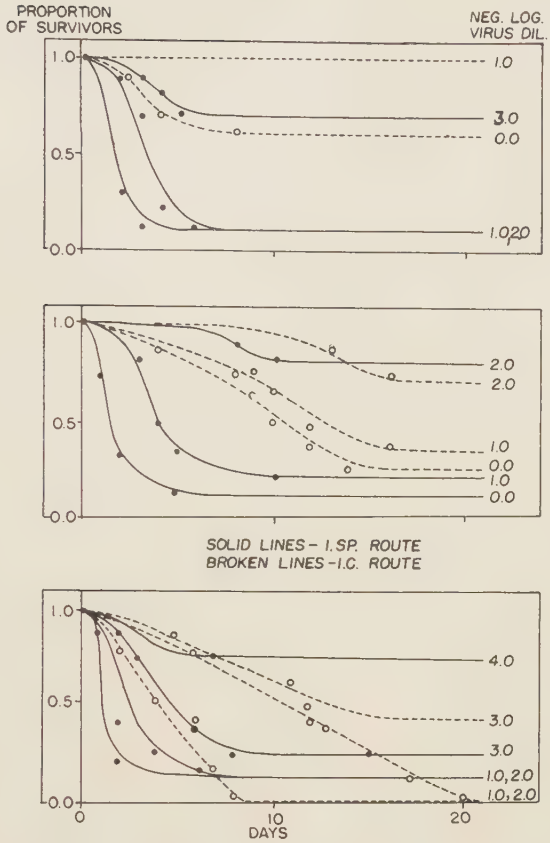

Figure 1 A
Leon 1 st. criss-cross passage

Figure 1 B
Leon 10th criss-cross passage

Figure 1 C
Lansing Pool
No. 062652

FIG. 1. Response time distribution for intracerebral and intraspinal route after the first and tenth criss-cross passage of mouse-adapted Leon virus as compared with the Lansing virus.

method developed by B. L. Bennett(5). *Tissue cultures.* We inoculated the Leon Strain into tissue cultures which were prepared according to a method developed by Dulbecco and Vogt(6). Cynomolgus monkey kidney mince was treated with 0.25% trypsin. The cell sediment was suspended in Synthetic Mixture 199 (Morgan, Morton and Parker) (7), giving a final concentration of cell sediment of 1/200. Two percent inactivated horse serum, 0.08% $NaHCO_3$ and penicillin and streptomycin were added to a final concentration of 100 units and 0.1 mg per ml respectively. The cultures in "milk-dilution bottles" were incubated at 36°C in a horizontal position and without motion for 6 days with a medium change after the 3rd day. Usually a complete tissue sheet covered the bottom of the bottles after 6 days. Before the inoculation of the virus the medium was changed again, but no horse serum was added. The final dilution of the virus in the inoculated vessel was $10^{-2.5}$. The cultures were kept at 36°C and harvested as soon as all tissue was degenerated. This degeneration usually occurred between the 6th and 8th day. By this method, fluids with high infectious titers could be obtained in sufficient quantities. It might be mentioned at this point that the mouse adapted Leon Strain behaved somewhat differently in tissue cultures when compared with certain other strains of each of the three types that were well established in this medium. The latter strains produced complete tissue degeneration between the 2nd and 4th day; whereas the mouse adapted Leon Strain showed a longer incubation period of 6-8 days. It is of further interest that undiluted supernatant fluid from a 10% cord suspension from Leon paralysed mice was

TABLE I. Proportion Responding (Paralysis or Death) for Both the Intracerebral and Intraspinal Route of the Leon Strain after Various Criss-Cross Passages. Adapted Lansing strain for comparison.

		Negative logarithms of virus dilutions inoculated:									
		Intracerebrally					Intraspinally				
Strain	Passage	0	1	2	3	4	0	1	2	3	4
Leon (Type 3)	1	4/10	0/10	0/10	0/10	—	9/10	9/10	3/10	0/10	—
	3	9/10	1/10	0/10	0/10	—	9/10	10/10	10/10	3/10	—
	8	10/10	10/10	2/10	0/10	—	10/10	9/10	6/10	0/10	—
	10	7/ 8	6/ 8	2/ 8	0/ 8	—	9/10	8/10	2/10	1/10	—
	13	10/10	10/10	9/10	6/10	—	11/11	11/11	10/10	9/10	1/10
Lansing (Type 2)		—	8/ 8	8/ 8	5/ 8	0/8	—	7/ 8	7/ 8	6/ 8	2/ 8

inoculated into tissue culture and produced no cytopathogenic effect over an observation period of 10 days.

Results. Fig. 1 shows the proportion of survivors when plotted against time in various titrations by the intraspinal as well as by the intracerebral route for type 2 and 3 poliomyelitis virus. Similar results were obtained when these fluids were titrated on different occasions, and this Figure illustrates the results of a typical experiment. A comparison of Fig. 1,A and 1,B shows clearly the shift of the response-time distribution of the intracerebral route towards that of the intraspinal one; this occurred between the 1st and 10th criss-cross passages. Thus this line of the Leon Strain is beginning to behave like the well adapted Lansing Strain (Fig. 1,C). It is of interest to note in Fig. 1,A the short incubation periods in the responding animals with early truncation of the curve for the intracerebral inoculated animals. This observation was consistent for material from the early passages. The behavior of the later passages is as shown in Fig. 1,B.

The proportion of responding animals for various virus dilutions tested is recorded in Table I, which shows another measure of adaptation. A rough estimate gives a potency† of intracerebral as compared with intraspinal route in the first passage of 0.01 while in the 10th passage it is about 1; correspondingly, the intracerebrally adapted Lansing Strain shows a relative potency of 1. A marked change of the relative potency occurred somewhere between the 3rd and 8th criss-cross passage.

Summary. The intracerebral adaptation of the Leon Strain of type 3 poliomyelitis to mice has been described.

† Relative potency, as commonly defined in bioassay, is the ratio of equally effective doses. Its logarithm may be taken as a difference between negative logarithms of two RD_{50} values, where RD_{50} is defined as the dilution estimated to give a 50% response (paralysis or death).

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Effect of Histamine, Epinephrine and Anti-Histamine (Antistine) on Glucose Turnover in the Isolated Liver. (20877)

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The clinical observation that certain antihistaminics induce a lowering of the blood sugar level(1,2) has led us to investigate the mechanism of this action. Since, as shown previously(3), this hypoglycemic effect can be obtained in the depancreatized and the alloxanized experimental animal, it does not seem to be mediated through the insulin-producing islet cell apparatus. We have therefore considered the possibility of a direct hepatic action and have now studied the effect of an antihistaminic on the carbohydrate metabolism of the isolated surviving liver. In addition, we have carried out similar experiments with histamine, partly in order to investigate the histamine-antihistamine relationship in this experimental setting, partly because the available data on the effect of histamine on carbohydrate metabolism are contradictory (4-8).

Material and procedures. Experiments were carried out with isolated and perfused liver of the bull frog (*Rana catesbiana*). The batrachian liver, if perfused with amphibian Ringer solution (NaCl 0.66, KCl 0.015, $CaCl_2$ 0.015, $NaHCO_3$ to pH 7.8, aqu. dest. ad 100) with addition of O_2 and glucose survives for several hours without spontaneous glycogenolysis. Bullfrogs, weighing 200 to 250 g, were kept in hibernating conditions at 4°C; they were killed by pithing. Livers were removed with preservation of proximal portions of the abdominal vein and the venous sinuses. Organs were weighed before and after the experiment; a slight weight gain of about 10% due to fluid retention occurred in most instances. The perfusions were performed as described previously(9) in accordance with the original method of Froehlich and Pollack (10) and the modifications of Geiger and Loewi(11) as to rate of flow and pressure. The perfusate entered the organ through the abdominal vein and was collected from the venous sinuses. The pressure was fixed at 20 cc H_2O. Recovery rate of the perfusate at start of experiments was set at 6-7 drops per minute. The returning perfusate was collected at 10-minute intervals and its glucose concentration was determined with the Nelson modification of the Folin-Wu micro method(12). An initial period of perfusion with an O_2 saturated amphibious Ringer solution served to wash out any free blood and blood glucose. After 20-30 minutes, the fluid usually returned water-clear and was sugar-free. At 40 minutes the perfusion fluid was changed to O_2 saturated amphibious Ringer-glucose solution, with a glucose concentration of 50 mg %. Within 10 or 20 minutes glucose content of returning fluid levelled off at the concentration of the entering perfusate. At this point the substances under investigation were injected into the perfusion system near the liver. The following substances were injected: 1) Histamine 0.1 mg, using histamine diphosphate (Abbott), 2) an antihistaminic drug, Antistine (Ciba), the dose being 5 mg, 3) Epinephrine 1 cc, 1:1,000,000.

Results. In a series of control experiments, no drugs were added to the perfusate; they confirmed previous observations(9) that after an initial period glucose concentration of returning fluid equalizes to that of the entering perfusate and remains at this level for periods of 2 to 3 hours. The results of experiments with drugs are demonstrated in Fig. 1. Six different experiments, each in duplicate, are shown. Since the main experiments started only after perfusion had been stabilized to the level of 50 mg % glucose, the initial periods with their fluctuations of the glucose concentration are not shown. The dotted lines on each graph represent the control level as established in the control experiments. It can be seen that histamine (0.1 mg) as well as epinephrine 1 cc (1:1,000,000) caused a marked

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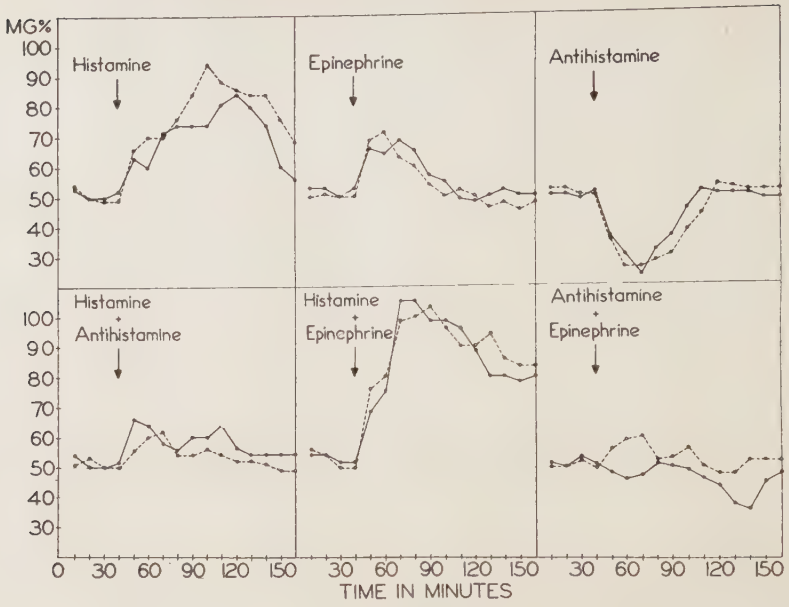

FIG. 1. Glucose concentrations of perfusates returning from isolated liver of bull frog, perfused with oxygenated amphibian Ringer solution containing 50 mg % glucose to which the following substances are added: Histamine 0.1 mg; epinephrine (1 cc—1: 1000000); antihistamine (Antistine) 5 mg; histamine + epinephrine, and Antistine + epinephrine. Two experiments shown in each instance. Initial periods of experiments during which livers were perfused without glucose are not included. Control level indicated in each graph by dotted line.

and transitory increase of the glucose concentration in the returning perfusate, while the antihistaminic caused an equally significant and transitory decrease. It must be emphasized that during these experiments, the rate of the perfusion remained grossly unaltered and that approximately the same amounts of fluid were collected during 10-minute periods before and after the addition of the test material. The second group of experiments shows the effect of simultaneous administration of Histamine + Antihistamine, Histamine + Epinephrine, and Antihistamine + Epinephrine in the above mentioned dosages. The antihistaminic is seen here to inhibit both the effect of histamine and that of epinephrine. Epinephrine and histamine, on the other hand, appear to have mutually augmenting effects; the increment of increase in the glucose content of the returning perfusate is not only greater but also more prolonged than if either substance is injected separately.

The last group of experiments (Fig. 2) was carried out to study the effect of antihistamine on spontaneous glycogenolysis of the surviving liver. The organ was perfused with a non-oxygenated sugar free amphibian Ringer solution. We have shown previously (9) that under these circumstances spontaneous glycogenolysis starts after 30 to 40 minutes of perfusion, and that this glycogenolysis is enhanced by epinephrine. This effect of epinephrine is demonstrated in the lower graph of Fig. 2. After spontaneous glycogenolysis had developed during the first 90 minutes of the experiment, epinephrine was added to the perfusate and a marked transitory rise in the glucose concentration of the returning fluid was found. The upper graph of Fig. 2 shows that the epinephrine effect was not only prevented by simultaneous injection of the antihistaminic, but that the glycogenolytic process itself was inhibited significantly. The decrease in the glucose concentration of the returning fluid indicates that glucose was retained in the organ, since the flow rate of the perfusion remained unchanged and no excessive amount of fluid was retained, as checked by the weight of the liver.

Comment. Our findings demonstrate that histamine and the antihistaminic substance used (Antistine) have direct effects upon the

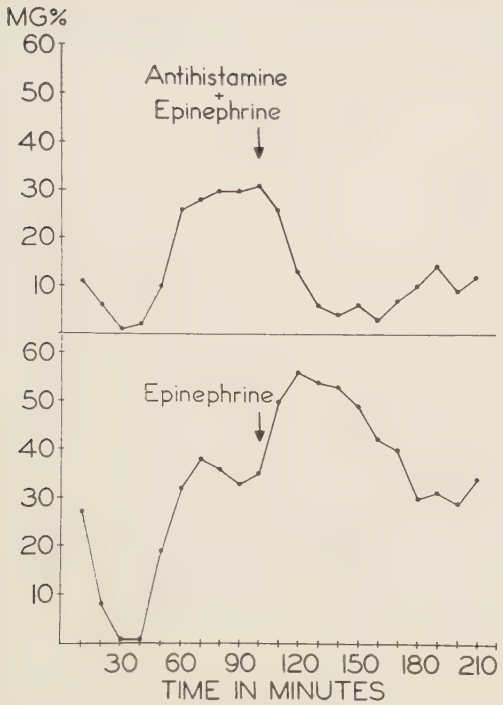

FIG. 2. Effects of epinephrine and of antihistamine (Antistine) plus epinephrine on glucose concentration of perfusate returning from isolated liver of bull frog perfused with amphibian Ringer solution. Epinephrine increases the spontaneous glycogenolysis; Antistine inhibits effect of epinephrine and diminishes spontaneous glycogenolysis.

carbohydrate metabolism of the surviving isolated batrachian liver. These effects are antagonistic inasmuch as histamine releases glucose from the hepatic tissue and the antihistaminic causes the hepatic tissue to retain glucose from the perfusate. With regard to the glycogenolytic action of epinephrine, it appears that histamine acts as a synergistic, while the antihistaminic acts as an antagonist. The observed effects seem to be the result of metabolic changes in the liver cells proper and not of changes in the vascular bed. Brauer and co-workers(13) have recently called attention to the vasomotor responsiveness of the isolated and perfused liver. In our experiments, however, pressure and rate of flow remained grossly unaltered, almost identical quantities of perfusate were collected during each fixed interval of time. The change in the glucose concentration of the perfusate would therefore seem to be caused by alterations in the cellular metabolism, *i.e.*, changes in intra-cellular glucose turn-over. Our findings tend to corroborate the observation of Chambers and Thompson(4), that histamine causes a decrease in hepatic glycogen. They are also in agreement with those investigators who reported a hyperglycemic phase after histamine administration(7,8). Furthermore, the demonstrated inhibition of hepatic glycogenolysis by the anti-histaminic used, suggests a possible mechanism for the clinically observed hypoglycemia induced by such substance.

Summary. The isolated liver of the bull frog is perfused with oxygenated amphibian Ringer glucose solution. The glucose concentration of the perfusate returning from the liver is determined in control experiments and under the influence of histamine, anti-histamine (Antistine) and epinephrine. Histamine and epinephrine cause an increase of the glucose concentration, probably due to enhanced glycogenolysis. Antistine causes a retention of glucose in the hepatic tissue. Antistine inhibits the spontaneous hepatic glycogenolysis as well as the glycogenolysis induced by histamine and epinephrine.

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A Rapid, Simplified Method for Serial Blood Volume Determinations in the Rat.* (20878)

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The extensive use of the rat in experimentation has emphasized the value of having a rapid, simple method of blood volume determination. The desirability of such a procedure was recognized as early as 1905 by Jolly and Stini(1) who employed the Welker Method. Scott and Barcroft(2) in 1924 developed a technic using carbon monoxide. The application of these methods was limited, however, because they required the sacrificing of the animal. Cartland and Koch(3) in 1928 modified the Keith-Rowntree plasma dye method(4) for use in the rat and made possible, for the first time, repeated observations in the same animal. In the light of subsequent advancements, however, their procedure has now become unnecessarily tedious and involved. Further modifications have therefore been undertaken and a much simplified version using Evans blue dye and the spectrophotometer is presented here.

Method. After withholding food, but not water, for the preceding 12 hours, the rat is lightly anesthetized, weighed and fastened to a ratboard. Using a 22-gauge needle and a calibrated syringe, 0.9 cc of blood for the standard sample is withdrawn by means of cardiac puncture. The needle is left in place and exactly 0.1 cc of 0.5% Evans blue dye is injected directly into the ventricle. The syringe is rinsed until all of the dye is removed, and the gentle flushing is continued over a 2-minute period to prevent clotting. At the end of that time, a second 0.9 cc sample of blood containing the dye is withdrawn. Each specimen is placed in an appropriately numbered Wintrobe hematocrit tube and centrifuged at 3000 RPM for 30 minutes. Dry heparin is used in both syringes and tubes to prevent clotting. Both hematocrits are recorded, and exactly 0.3 cc of plasma is withdrawn from each hematocrit tube. All of the syringes used for injecting dye and withdrawal of plasma and blood are previously calibrated with mercury. The dye free sample to be used for the standard is diluted to exactly 3.0 cc with a 1:1000 solution of the 0.5% Evans blue dye in normal saline. The unknown sample containing the injected dye is also diluted to exactly 3.0 cc with normal saline giving it a dilution factor of 10. With the wave length setting at 620 mμ, the respective densities are then determined using the Beckman spectrophotometer. The total blood volume and blood volume per 100 g of body weight are determined using the following formulae:

$$PV = \frac{D_1}{D_2} \times 10$$

PV = plasma vol; D_1 = density reading of standard; D_2 = density reading of plasma containing unknown amount of dye.

$$TBV = \frac{PV}{1 - (.96 \times Hct.)} + .8$$

TBV = total blood vol; PV = plasma vol; Hct = hematocrit.

$$BV/100\ g = \frac{TBV}{\frac{\text{Wt of rat in g}}{100}}$$

BV = blood vol; TBV = total blood vol.

Results. In the first 100 determinations, the blood volume ranged between 4.00 and 6.43 cc/100 g body weight. An example of the findings is given in Table I. The average value of 5.04 cc was significantly lower than the findings of Cartland and Koch(3) (6.9 cc/100 g), Orten *et al.*(5) (6.38 cc/100 g), and Went and Drinker(6) (7.4 cc/100 g) who used vital red dye, and Metcalf and Favour(7) (7.6 cc/100 g), and Beckwith and Chanutin(8) (7.98 cc/100 g) who used Evans

* This work was done in the Department of Pathology, Yale University School of Medicine and was supported by the Office of Naval Research.

TABLE I. Example of Results Obtained in Blood Volume Determinations on 10 Different Animals.

Wt, g	Hematocrit	D_1	D_2	Plasma vol, cc	TBV, cc	BV/100 g, cc
400	.460	.348	.252	12.45	23.10	5.77
405	.476	.363	.314	10.04	19.24	5.04
390	.480	.365	.294	11.20	21.40	5.49
366	.490	.312	.288	9.75	18.50	5.29
421	.500	.333	.263	11.40	21.90	5.39
416	.505	.360	.310	10.48	22.03	5.30
403	.554	.370	.336	9.94	22.10	5.55
326	.440	.497	.470	9.53	17.30	5.31
432	.515	.355	.282	11.35	23.23	5.36
468	.500	.344	.254	13.58	27.18	5.81

blue dye. It does, however, compare favorably with those found by Griffith and Campbell(9) (4.3 cc/100 g) who used vital red dye, and by Berlin and his associates(10) who used P^{32} (3.94-5.95; avg 4.59 $\pm$ 0.57 cc/100 g) and Fe^{59} (3.63-5.81 cc/100 g). Although those methods employing radioactive tracers are at present too intricate for mass application, they are probably the most accurate that are currently available.

The use of intracardiac puncture was not particularly hazardous. The mortality rate for the first 148 determinations was 4.05%. All of the animals were eventually sacrificed and at autopsy only a focal organized pericarditis was present over the injection site.

Discussion. Depending upon the individual needs, several satisfactory methods for determining the blood volume of the rat have been devised. When volumetric studies are required on large numbers of animals, however, they have proven to be too elaborate in their procedure and not readily applicable to mass production. With reasonable attention to detail, the method described above has been found to be relatively simple and accurate, and with planning as many as 15 animals can be processed during one determination period. It is also useful when serial determinations are required. An example of these findings is given in Table II.

TABLE II. Example of Comparative Results of Serial Determinations Obtained at Time Intervals Varying from 1 Week to 3 Months on 10 Different Animals.

Determinations		
1st	2nd	3rd
4.34	4.74	4.35
5.75	5.32	5.78
5.14	5.15	5.16
4.47	4.74	5.05
4.15	4.68	4.67
4.13	4.73	4.14
5.15	5.45	5.18
4.65	4.39	4.65
4.87	5.76	5.28
4.34	4.39	4.42

The usual sources of error native to this type of procedure were controlled in the following manner: 1) the 2-minute time interval between injection of the dye and withdrawal of the final sample was sufficient to permit complete mixing of the material without permitting significant extravascular loss; 2) as the absorption of hemoglobin is negligible in the maximum spectral absorption range of Evans blue dye (620 mμ), slight hemolysis was of no consequence and 3) the problem of lipemia was eliminated by diet.

Summary. A relatively rapid, simplified method for the determination of the blood volume in the rat using Evans blue dye and the spectrophotometer is described. It has been found to be satisfactory for both mass production and serial determinations. A comparison of the results with those obtained by other methods is given.

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Interaction of Heparin Active Factor and Egg-Yolk Lipoprotein.* (20879)

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The *in vivo* action of heparin on plasma lipids was initially observed by Hahn to be a clearing of a lipemic plasma of its light scattering components(1). Such action definitely suggested a transformation of the physical state of the plasma lipids. The work of Gofman and co-workers at this laboratory has shown that the different plasma lipids measured chemically could be accounted for in the spectrum of lipoprotein molecules(2). The action of heparin on the lipemic plasma indicated its probable involvement with this whole spectrum of lipid-bearing molecules. The action of *in vivo* heparin on the spectrum was observed by Graham and co-workers and subsequently studied by Lindgren and co-workers at this laboratory(3,4). Their investigations bring forth the observation that subsequent to an injection of heparin there is a uni-directional transformation of lipid-bearing molecules from the ultracentrifugally defined high S_f (molecules of high glyceride content) to the lower S_f values (which are correspondingly lower in glycerides). Such transformations could be produced *in vitro* by incubation of a lipemic serum with *in vivo* heparinized plasma. This report presents an investigation of the interaction of the active principle or factor of heparinized plasma with lipoprotein species isolated from egg yolk(5). The interaction was manifested by a reduction in the optical density of a solution containing a small amount of ELP (egg lipoprotein) maintained at a low ionic strength and pH 6.7. Addition of heparin in low concentrations to the dilute ELP solution increased the optical density. Thus, there was present in post heparin plasma an agent which altered the solubility characteristics of the ELP solution and which could not be reproduced by a simple *in vitro* addition of heparin.

Methods. The ELP was obtained by means of a 12-hour preparative ultracentrifugation at 30,000 RPM (Spinco preparative centrifuge and 30.2 rotor) of egg yolk contents diluted in the proportion one yolk to 18 ml phosphate buffer (pH 8.0; ionic strength 0.1). The lipoprotein forms a yellow concentrate in the uppermost zone of the centrifuge tube. This concentrate is carefully removed and when diluted one to 4 parts with the above buffer serves as a stock solution (concentration approx. 5%). An analytical ultracentrifugation (in density 1.0630 at 26°C at 52,640 RPM) determines the concentration and any sedimenting contamination, which is to be avoided, in the stock solution. Under the above conditions the lipoprotein preparation has a major component of approx. 25-30 S_f units with some faster species generally present. Infra-red chemical analyses by Freeman(6) give an average composition for the preparation as follows: Glyceryl ester, 67%; cholesterol (predominantly free), 6%; phospholipid (as lecithin), 16%; protein, 11%. For measurements on the interaction system of ELP with either pre-heparin serum‡ or post-heparin plasma§ the technic was to incubate

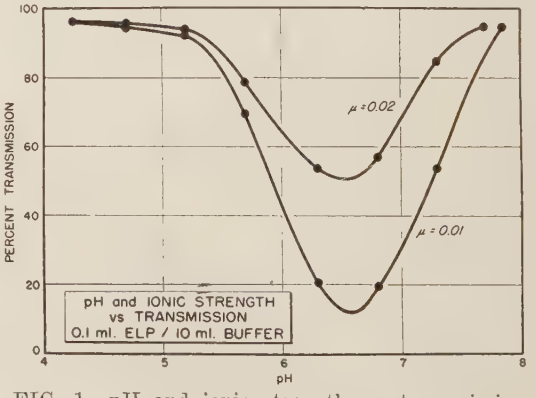

FIG. 1. pH and ionic strength vs. transmission. 0.1 ml ELP/10 ml buffer.

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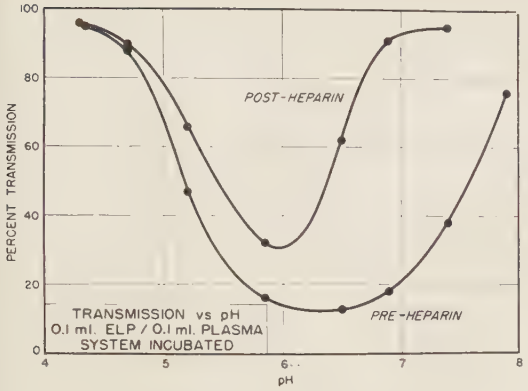

FIG. 2. Transmission vs. pH. 0.1 ml ELP/0.1 ml plasma, system incubated.

0.1 ml of a 3 to 5% ELP solution with 0.1 ml of serum or plasma at 37°C for a specified time. At the end of the incubation period the mixture was diluted with 10 ml of an appropriate buffer (depending on required pH value), stirred, and allowed to stand for 15 minutes. After this period the diluted mixture was stirred and the optical transmission at 650 mμ (Beckman spectrophotometer) was measured. The pH of the incubation mixture was controlled around pH 8.0 in order to operate in the neighborhood of maximal increase in transmission as shown in Fig. 5.

Results. As shown in Fig. 1 the ELP solubility is increased by an increase in ionic strength and is at a minimum in the region of pH 6.7. Fig. 2 shows that at a constant ionic strength of 0.01, there is a marked difference in solubility, when the effects of pre-heparin serum and post-heparin plasma on the ELP are compared. The shift in the point of minimum solubility, or what may be expressed alternatively, the increased solubility of the ELP in a given region of pH, at ionic strength 0.01, was observed only if one or more of the following conditions were met: 1) the post-heparin plasma was obtained from an individual whose blood contained an elevated level of lipoprotein species of high glyceride content. 2) the post-heparin plasma, obtained from a fasting individual normally low in the glyceride-containing lipoprotein species, was incubated with a lipemic serum prior to the addition of ELP. 3) a fasting post-heparin plasma, again from an individual low in glyceride-containing lipoproteins, was incubated with ELP before testing for activity. This condition is illustrated by comparison of Fig. 3 with Fig. 2 (incubated versus non-incubated systems in which plasma came from a fasting individual low in glyceride-containing lipoproteins). Thus, an essential requirement for the production of the shift in ELP solubility was a *prior* interaction—either *in vivo* or *in vitro* at 37°C—of heparinized plasma with lipoprotein species high in glyceride content.

The necessity for the presence of lipoproteins high in glycerides to enable post-heparin plasma to increase the solubility of ELP indicated that there may be the release of a labile lipid constituent which itself may effect the alteration. Such a constituent does not alter the pH of the system to a significant degree under the conditions of the solubility determination. Therefore, it might be supposed that a soluble agent with a very high surface activity such as a fatty acid soap is released during the interaction. Fig. 4 shows the effect of sodium oleate on ELP, and that it is analogous to the post-heparin plasma interaction effect shown in Fig. 2.

Data from rate determinations are shown in Figs. 6 and 7. The reaction was stopped with the addition of 10 ml of cacodylate buffer (ionic strength 0.01; pH 6.7). A plot

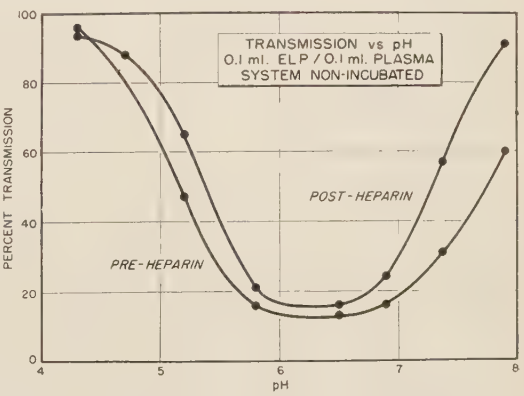

FIG. 3. Transmission vs. pH, 0.1 ml ELP/0.1 ml plasma, system non-incubated.

‡ Pre-heparin serum obtained from untreated whole blood from an individual prior to intravenous administration of heparin.

§ Post-heparin plasma obtained from whole blood drawn from an individual after intravenous administration of heparin.

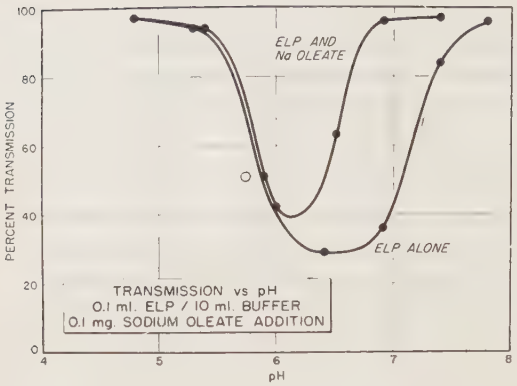

FIG. 4. Transmission vs. pH. 0.1 ml ELP/10 ml buffer, 0.1 mg sodium oleate addition.

of transmission versus varying concentrations of ELP in 0.1 ml in the presence of 0.1 ml preheparin serum is also shown in Fig. 6. This plot was obtained by adding 10 ml of the cacodylate buffer to the volumes of ELP and serum stated above, with no incubation. The transmission at any time interval was compared with a transmission obtained from the above concentration plot. Thus, the fraction of ELP still insoluble after an interval of incubation as evidenced by the % transmission is compared with a concentration of ELP necessary to give the same % transmission. By this procedure the rate curves plotted as changes in transmission against time can be reduced to comparatively linear curves expressed as concentration changes. Calibration is also possible with sodium oleate.

The duration of plasma activity *in vivo* after a single 50 mg intravenous injection of heparin as measured under the conditions already described for rate studies, is shown in Fig. 8. Within 3 hours after the heparin injection the serum of this subject no longer had any demonstrable effect. Following intravenous injections of 100 mg the effect was observed to persist for 4 to 5 hours. Again, in Fig. 8, it should be noted that measurement of transmission without prior incubation of the ELP with the plasma reveals no change following the heparin injection because of the initial low level of glyceride in this subject's serum. Further chemical investigations at this laboratory have substantiated this hypothesis by showing that fatty acid is indeed liberated with a concomitant decrease in triglycerides when lipoproteins high in glyceride content are incubated with postheparin plasma(7). Further studies are in progress toward establishing the type of reaction scheme in the above system and its dependence on the concentration of the reactants.

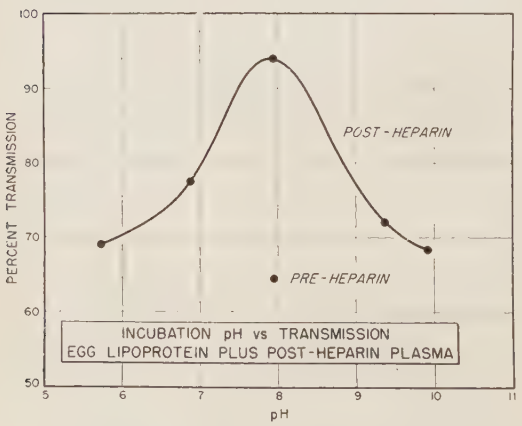

FIG. 5. Incubation pH vs. transmission. Egg lipoprotein plus postheparin plasma.

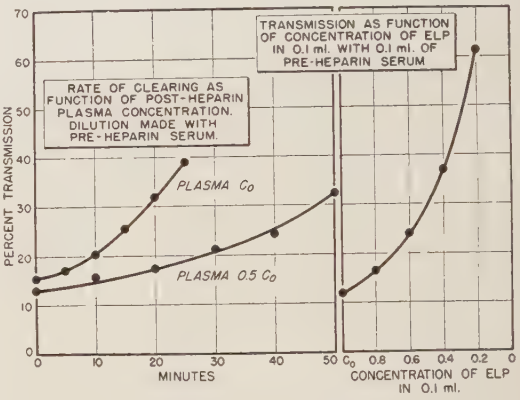

FIG. 6. Transmission as function of concentration of ELP in 0.1 ml with 0.1 ml of preheparin serum. Rate of clearing as function of postheparin plasma concentration. Dilution made with preheparin serum.

Undiluted postheparin plasma is designated C_o conc.; any dilution thereof with preheparin plasma or serum is designated as a fraction of C_o. Egg lipoprotein (ELP) stock sol. (whose actual conc. is obtained by analytical ultracentrifugation) is also designated C_o conc. and any dilution thereof with appropriate buffer is a fraction of that C_o conc.

Summary. 1. Egg lipoprotein under the conditions stated can be used as a qualitative

and semi-quantitative indicator for the lipoprotein-influencing properties of *in vivo* heparinized plasma. 2. The utilization of egg lipoprotein for this purpose requires prior contact of the active principle of post-heparinized plasma with lipoproteins of high neutral fat content. 3. Sodium oleate produces alterations in the egg lipoprotein system similar to those produced by post-heparin plasma. Chemical data showing that fatty acids are released from the glycerol ester fraction of the egg lipoprotein during incubation suggest that the egg lipoprotein solubility changes may be a secondary effect of the hydrolysis of glycerol esters by the active principle in post-heparinized plasma.

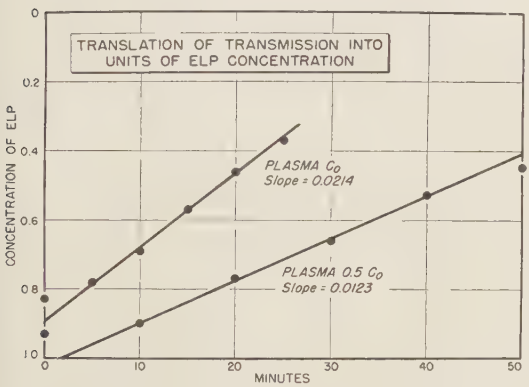

FIG. 7. Translation of transmission into units of ELP concentration.
Undiluted postheparin plasma is designated C_o conc.; any dilution thereof with preheparin plasma or serum is designated as a fraction of C_o. Egg lipoprotein (ELP) stock sol. (whose actual conc. is obtained by analytical ultracentrifugation) is also designated C_o conc. and any dilution thereof with appropriate buffer is a fraction of that C_o conc.

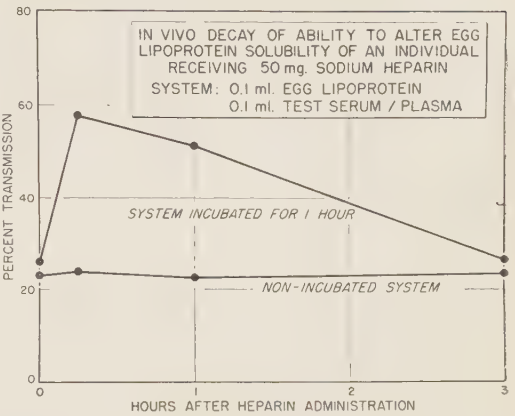

FIG. 8. *In vivo* decay of ability to alter egg lipoprotein solubility of an individual receiving 50 mg sodium heparin. System: .1 ml egg lipoprotein, .1 ml test serum/plasma.

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Effect of Heparin on Fate of Intravenously Administered Fat Emulsions in Rats.* (20880)

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From the Army Medical Nutrition Laboratory, Chicago, and the Department of Clinical Science, University of Illinois.

The injection of heparin is known to produce clearing of the turbidity of plasma associated with alimentary lipemia(1). A previous report from this laboratory(2) described experiments showing that plasma from rats which had received an intravenous injection of heparin was capable of clearing *in vitro* the turbidity caused by addition of fat emulsion to the plasma.

The present study was undertaken to determine what effect simultaneous injection of heparin and fat emulsion would have on the rate of disappearance of fat from the bloodstream and on the uptake of fat by various organs. Several related phenomena were also investigated.

Methods. The fat emulsion used in this study was prepared by and kindly supplied by Dr. Douglas Frost and his associates of Abbott Laboratories, North Chicago, Ill. It contained 10 g of sesame oil, 0.5 g of Tween 60 and 5 g of glucose per 100 ml. It was prepared by high pressure homogenization and the particle size was one μ or less. Forty-eight male Sprague-Dawley rats weighing between 200 and 250 g received a single rapid injection of 33 ml/kg of this emulsion into the tail vein. One-half of the animals received 10 mg/kg of heparin (Abbott) intravenously simultaneously with the fat emulsion. Three rats from the heparin-treated group and 3 from the group without heparin treatment were sacrificed at each of the following intervals after injection: 5, 10, 20, 40, 80, 120, 240, and 480 minutes. At the time of sacrifice blood samples were drawn from the abdominal aorta under ether anesthesia. The liver and spleen were removed for total lipid analysis by the method of Geyer *et al.* (3). The total esterified fatty acid concentration of the blood serum was determined by the method of Bauer and Hirsch(4). Six rats which had not been injected with fat emulsion were used to obtain control values. In the studies on hemolysis, plasma or serum hemoglobin was determined by the method of Flink and Watson(5). Before performing hemoglobin determinations on lipemic plasma or serum samples these were clarified by shaking with an equal volume of chloroform, centrifuging and separating the supernatant plasma.

Results. Fig. 1 presents the results of serum esterified fatty acid determinations at various intervals after injection of fat emulsion with and without heparin. Each point on the graph represents the mean value of determinations on 3 rats. The blood lipid levels were approximately the same in the 2 groups of animals during the first 10 minutes after injection but thereafter the group which received heparin showed a much more rapid return to control levels. As early as 20 minutes after injection there was a striking

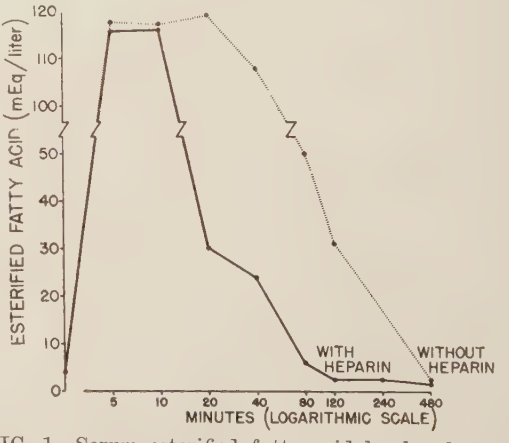

FIG. 1. Serum esterified fatty acid levels of rats at various times after intravenous injection of 33 ml/kg of fat emulsion with and without 10 mg/kg of heparin.

* The opinions expressed in this paper are those of the authors and do not necessarily represent the official views of any governmental agency.

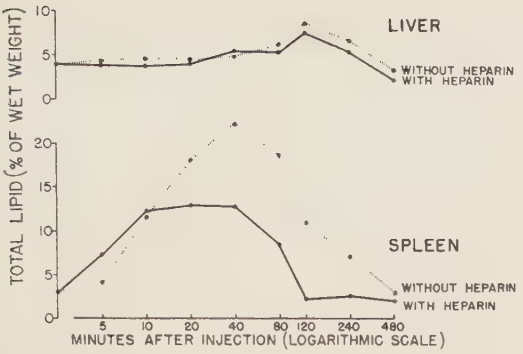

FIG. 2. Total lipid concentration of liver and spleen of rats at various intervals after intravenous injection of 33 ml/kg of fat emulsion with and without 10 mg/kg of heparin.

difference in blood fat levels. The centrifuged blood specimens at the 40-minute interval from the group which did not receive heparin showed marked turbidity whereas those from the group which received heparin showed no turbidity in spite of the fact that the serum fat level of the latter group was still substantially elevated above the control value at this time. This indicates that loss of turbidity proceeds more rapidly than removal of fat from the bloodstream but that both processes are accelerated by heparin.

Figure 2 depicts the results of the total lipid determinations on the spleens and livers. In the spleen the lipid concentration rose progressively during the first 40 minutes and then gradually returned to control values. In the group of rats which received heparin the rise in lipid concentration in the spleen was smaller and the return to the control level was more rapid. The magnitude of the uptake of the injected fat by the spleen is striking. In a separate study the mean weight of the spleen was found to increase from a control value of 2.2 g/kg body weight to 3.7 g/kg one hour after intravenous injection of 33 ml/kg of fat emulsion. The mean per cent of injected fat recovered from the spleen at this time was 30%. In the liver (Fig. 2) the increase in lipid concentration takes place much later after fat injection than in the case of the spleen and is much smaller in magnitude. However, since the weight of the liver is roughly 10 times that of the spleen a given percentage rise in lipid concentration in the liver represents 10 times as much fat on an absolute basis. The increase in liver lipid concentration takes place at a time when the spleen lipid concentration is rapidly decreasing. Although the lipid concentration in the liver is lower in the animals which received heparin, the difference is small.

In the course of the studies on the rate of disappearance of fat from the blood after intravenous injection it was noted that the lipemic samples always showed considerable hemolysis. A study was made of the effect of heparin injection on the degree of hemolysis in lipemic sera. A study also was made to determine whether alimentary lipemia would produce hemolysis. The results of this study appear in Table I and show that both oral administration and intravenous injection of fat lead to hemolysis. The lesser degree of hemolysis seen in the plasma of the rats given the fat emulsion orally as compared with those given the emulsion intravenously is probably due to the lower fat concentration in the serum of the former group. The results of the experiments in which heparin was given together with fat emulsion intravenously appear in Fig. 3. The values in the graph are the means of determinations on sera from 3 animals in each group. It will be noted that marked and equal elevation of plasma hemo-

TABLE I. Serum Hemoglobin and Lipid of Rat Blood after Oral or Intravenous Administration of Fat Emulsion.

No. of rats	Treatment	Esterified fatty acids (mEq/l)	Serum hemoglobin (mg %)
10	Untreated controls	4.64 ± .78	7.17 ± 1.64
12	10 ml/kg fat emulsion by stomach tube 4 hr before drawing blood	14.61 ± 1.29	86.5 ± 9.58
10	10 ml/kg fat emulsion intrav. 5 min. before drawing blood	118.12 ± 14.17	427.7 ± 4.42

Figures following mean values are stand. errors.

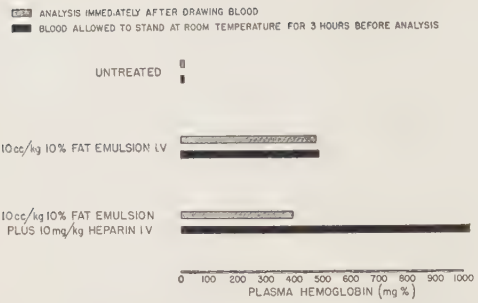

FIG. 3. Plasma hemoglobin of rats 5 min. after intravenous injection of fat emulsion with or without heparin.

globin occurred in both the group which received fat emulsion with heparin and the group which received fat emulsion alone. However, when the blood samples were permitted to stand at room temperature for 3 hours a marked further increase in plasma hemoglobin occurred in those from the group which had received fat emulsion plus heparin, whereas no change was seen in the samples from the group which had received only fat emulsion.

Discussion. The results of this study indicate that heparin is capable of producing clearing of lipemia induced by injection of fat emulsions in the same manner that it has previously been shown to produce clearing of alimentary lipemia. Under the influence of heparin not only is the physical state of the fat in the blood altered so that turbidity is decreased but also the fat leaves the blood more rapidly. This extremely rapid disappearance of fat from the blood cannot be accounted for by increased rate of uptake of the fat by the liver or spleen, as the concentration of lipid in these organs is actually decreased. Since accumulation of fat in the spleen occurs after intravenous but not after oral administration of fat, by reducing splenic uptake of fat heparin causes intravenously injected fat to behave more nearly like orally administered fat.

Creditor(6) has shown that intravenous injection of fat emulsion produces a marked increase in mechanical fragility of red blood cells. Weld and Spitzer(7) found that the injection of heparin into dogs with alimentary lipemia produced a decrease in the antihemolytic activity of the plasma although lipemia alone caused no significant change. The question arises as to whether the elevation of serum hemoglobin seen in lipemic blood is due to *in vivo* hemolysis or whether it is due to hemolysis occurring during and after blood drawing as a result of increased red blood cell fragility. Although the studies of Johnson *et al.*(8-10) suggest that lipemia produces *in vivo* hemolysis, studies failed to show an elevation of biliary bilirubin excretion rate after intravenous injection of fat emulsion with or without heparin(11). We interpret our results to indicate that the elevated serum hemoglobin values occurring with lipemia following ingestion or injection of fat are due to *in vitro* hemolysis resulting from the increased fragility of red blood cells in the presence of fat. The intravenous injection of heparin apparently produces a delayed increase in the magnitude of this *in vitro* hemolysis. We and others(12) have found that free fatty acid is released when post-heparin plasma is incubated with a neutral lipid substrate. This release of free fatty acid might account for the increased hemolytic activity of post-heparin lipemic blood on standing at room temperature.

Conclusions. 1. Addition of heparin to intravenously administered fat emulsion in rats resulted in a marked increase in the rate of disappearance of the fat from the blood and a decrease in the accumulation of fat in the spleen and liver. Heparin apparently caused a change in the physical state of the fat in the blood resulting in decreased turbidity and this altered fat rapidly left the blood. 2. Lipemic blood from rats either fed fat or injected with fat emulsion showed elevated serum hemoglobin concentrations. Injection of heparin with fat emulsion resulted in higher serum hemoglobin levels than injection of fat emulsion alone but this difference was seen only when the blood was allowed to stand for a period after it had been drawn.

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Ultrafiltration and Electron Microscopy of Three Types of Poliomyelitis Virus Propagated in Tissue Culture.* (20881)

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Previous work on filtration of poliomyelitis virus through gradocol membranes(1-3) was performed with Type 2 strains (MV, Lansing) propagated in the nervous system of monkeys or mice, and the extracts or stock filtrates actually used in the critical filtration experiments contained as little as one and not more than 100 infective doses. Using Elford's formula and different methods of estimating the average pore diameter (APD) which held back the virus, the size was variously reported as 8-12 mμ(2), 10-15 mμ (1,4), and 15-23 mμ(3). Since filtration endpoints may be affected both by the concentration of virus and the presence of extraneous tissue particles, it appeared possible that the size of the poliomyelitis viruses might actually prove to be smaller in tests with water-clear tissue culture fluids containing 10^6 to 10^8 infective doses per ml. Poliomyelitis virus of all 3 immunologic types, propagated in cynomolgus kidney tissue cultures, was studied by filtration through gradocol membranes and by electron microscopy.

Materials and methods. With one exception, the virus used in these tests was grown in 250 ml centrifuge bottles in a roller drum. The washed, minced cynomolgus kidney tissue, suspended in chick embryo extract, was embedded over the entire inner surface of the bottles previously coated with chicken plasma. The medium consisted of 0.5% lactalbumin hydrolysate (enzymatic—Nutritional Biochemicals Corp., Cleveland), Simms ox serum ultrafiltrate, Earle's balanced salt solution, 0.05 mg of crystalline soybean trypsin inhibitor, 100 units of penicillin and 0.1 mg of streptomycin per ml; 40 ml of this medium were used per bottle. After 7 days in the roller drum at 35°C, the bottles were inoculated with 0.1 ml of undiluted, virus-containing tissue culture fluid. Virus was harvested twice at 2- or 3-day intervals. In some tests only the first harvest was used, in others a pool of the two. The virus was stored in the frozen state in a chest containing solid CO_2. Virus titrations were performed in roller tubes of cynomolgus kidney tissue cultures. The gradocol membranes were purchased from St. Mary's Hospital, London, England. Prior to filtration of the tissue culture fluid, brain and heart infusion, proteose-peptone broth (Difco) was passed through the membranes—10 ml for membranes with an APD of more than 100 mμ and 5 ml for those less than 100 mμ. Filtration was performed under positive pressure of nitrogen—10 lb per square inch for membranes over 100 mμ and 15 lb for

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those under 100 mμ. The tissue culture fluid was centrifuged at about 2,000 rpm for 10 minutes on an ordinary horizontal centrifuge. The Type 1 and Type 2 poliomyelitis virus culture fluids passed through the 220 mμ membranes within a few minutes without evidence of clogging and the resulting filtrate was the stock used for the other membranes. The Type 3 culture fluids, however, quickly clogged even 1140 mμ membranes after filtration of the first 15 to 20 ml. Accordingly in the last test with this virus, a separate 1140 mμ membrane was used for each 20 ml of tissue culture fluid. The 1140 mμ filtrate did not clog the 680 mμ membrane, but the 680 mμ filtrate still clogged the 290 mμ membranes and 4 of these membranes had to be used to collect 94 ml of stock filtrate. The virus titer of the 290 mμ stock filtrate was about the same as that of the unfiltered tissue culture fluid. The amounts filtered through the other membranes varied from 10 to 18 ml. The diameter of the effective membrane surface was 2.7 cm. The identity of the virus preparations used in these tests was confirmed by immunologic tests.

Results of ultrafiltration. The data presented in Table I indicate that, despite the high virus content of the tissue culture fluids, the estimated size of the 3 types of poliomyelitis virus turned out to be larger rather than smaller than the 8-12 mμ(2) and 10-15 mμ (1,4) reported in the tests with extracts of monkey spinal cords of very low potency. In 2 of the present tests, in which the virus failed to pass the 50 mμ membranes, the stock filtrates had titers of $10^{5.2}$ and $10^{5.4}$ TCD_{50} (50% tissue culture cytopathogenic doses). Passage through the 44 mμ, and in one instance the 39 mμ, membrane was obtained only with stock filtrates of much higher potency. In all the tests, except the last with Type 3 virus, the inoculum in each tube was 0.2 ml and the number of tubes used for each filtrate was only 4; in the last test with Type 3 virus, in which passage through the 39 mμ membrane was achieved, the inoculum was one ml per tube and 5 tubes were used for each filtrate. With the variations just noted, the filtration end-point for all 3 types of poliomyelitis virus appears to be about the same and, according

TABLE I. Gradocol Membrane Filtration of 3 Types of Poliomyelitis Virus Propagated in Kidney Tissue Culture.

Membrane used		Type 1 (Mahoney)		Type 2 (Y-SK)				Type 3 (Leon)			
		KP54-$10^{7.4}TCD_{50}$/ml		KP10-$10^{6.2}TCD_{50}$/ml		KP34-$10^{7.8}TCD_{50}$/ml		KP3-$10^{5.0}TCD_{50}$/ml		KP58-$10^{6.7}TCD_{50}$/ml	
APD, mμ	Thickness, mm	Amt filtered, ml	Result	Amt filtered, ml	Result	Amt filtered, ml	Result	Amt filtered, ml	Result	Amt filtered, ml	Result
290	.145									94	$10^{6.5}TCD_{50}$/ml
220	.174	62	1, 1, 1, 1*	55	$10^{6.2}TCD_{50}$/ml	80	1, 1, 1, 1	61	$10^{5.4}TCD_{50}$/ml		
79	.172					15	2, 2, 2, 3				
60	.151	15	1, 1, 1, 1			15	2, 2, 2, 2	10	2, 3, 6, 0	18	1, 1, 1, 1, 1
50	.158	15	2, 2, 2, 2	5	0, 0, 0, 0	15	2, 2, 2, 3	12	0, 0, 0, 0	18	1, 1, 1, 1, 2
44	.137	15	2, 2, 2, 2					10	0, 0, 0, 0	18	1, 1, 1, 1, 1
39	.123	15	0, 0, 0, 0			15	0, 0, 0, 0	13	0, 0, 0, 0	18	2, 2, 2, 2, 3
36	.116			12	0, 0, 0, 0					18	0, 0, 0, 0, 0
28	.092			12	0, 0, 0, 0	15	0, 0, 0, 0	13	0, 0, 0, 0		
Size based on Elford's formula		——13-20 mμ——		——13-20 mμ——				——12-18 mμ——			
Size of particles seen with electron microscope		30 mμ		27 mμ				41 mμ			

* Each figure refers to number of days after inoculation that a complete or almost complete cytopathogenic change was observed in one culture tube.

to Elford's formula, the size would be in the range of 12 to 20 mμ. It should be noted here that Elford, Galloway, and Perdrau(2) demonstrated unequivocal passage of virus only through membranes having an APD of 40 mμ or more, and based their estimate of size on the assumption that, if their virus preparations had been more potent, the membrane just failing to permit passage would have been 24 mμ. In the experiments of Theiler and Bauer(1) the virus passed regularly through membranes with an APD of 90 mμ or more, irregularly in the range of 67 to 40 mμ, and only once in 4 tests through a 35 mμ membrane, the filtrates infecting only one of 7 inoculated monkeys.

It appeared desirable, therefore, to determine by filtration through the same batch of membranes whether or not the same strain of virus propagated in the nervous system of monkeys might yield a certain proportion of particles that would be smaller than those produced in kidney tissue culture. A 20% uncentrifuged, frozen suspension of cynomolgus spinal cords, containing the Y-SK virus that initiated the kidney tissue cultures used in the present tests, was diluted to 1% with the tissue culture medium previously described. After centrifugation at 10,000 rpm in the No. 30 Spinco head (8,700 g) for 30 minutes in the cold and *in vacuo,* 96 ml of the supernatant liquid passed through a single 680 mμ membrane without any evidence of clogging in about 2 minutes. This filtrate then passed through a single 290 mμ membrane in about 8 minutes and the resulting stock filtrate was used for all the other membranes. The results shown in Table II are based on tests in kidney tissue culture tubes inoculated with one ml amounts of filtrate. Comparative titrations of this virus in monkeys and kidney tissue culture tubes indicated that the *in vitro* method may even be slightly more sensitive. The irregular results, which are similar to those reported by Theiler and Bauer(1), do not permit the conclusion that poliomyelitis virus propagated in the nervous system of monkeys can be smaller than that developing in tissue culture.

Observations with the electron microscope. Preparations from the following lots of tissue culture fluids were studied: a) *Control uninoculated fluid* consisting of a pool of 3 consecutive harvests made 11, 14, and 18 days after preparation of a large kidney tissue culture bottle in which medium No. 199 was used instead of the lactalbumin hydrolysate medium previously described; b) *Type 1 (Mahoney), kidney passage 30,* consisting of a pool of 2 successive harvests, which had $10^{7.9}$ TCD_{50} of virus per ml, the lactalbumin hydrolysate medium being used here; c) *Type 2 (Y-SK), kidney passage 10,* consisting of a pool of 2 successive harvests, which had $10^{6.2}$ TCD_{50} of virus per ml, the medium being No. 199 and an aliquot of this lot being used in the gradocol membrane filtration test; and d) *Type 3 (Leon), kidney passage 3,* consisting of a pool of 2 successive harvests, which had $10^{6.2}$ TCD_{50} of virus per ml, the medium being lactalbumin hydrolysate and an aliquot of this lot, twice diluted with Hanks' balanced salt solution, being used in the gradocol membrane filtration test. Each of these pools was centrifuged at low speed and stored in the frozen state in a chest containing solid CO_2 for several weeks before the specimens were prepared for electron microscopy.

TABLE II. Gradocol Membrane Filtration of Type 2 Poliomyelitis Virus (Y-SK) Propagated in Nervous System of Cynomolgus Monkeys. Titer of unfiltered virus = $10^{6.2}TCD_{50}$/g or $10^{4.2}TCD_{50}$/ml of 1% suspension.

APD of membrane, mμ	Amt filtered, ml	Time of filtration, min.	Result
290	96	8	$10^{3.5}TCD_{50}$/ml 2, 2, 2
60	12	20	6, 11, 0, 0, 0
50	20	50	0, 0, 0, 0, 0
44	20	50	3, 4, 6, 7, 10
39	20	60	6, 0, 0, 0, 0
36	20	74	0, 0, 0, 0, 0
Size based on Elford's formula			12-18 mμ

A total of 111 ml of each fluid was distributed in 3 chilled, stainless steel centrifuge tubes and spun at 8,700 g for 30 minutes in a chilled Spinco No. 30 rotor. A barely perceptible sand-gray sediment was present after the supernatant liquid was decanted. This supernatant liquid was again centrifuged in a cold Spinco No. 30 rotor at 70,000 g for one

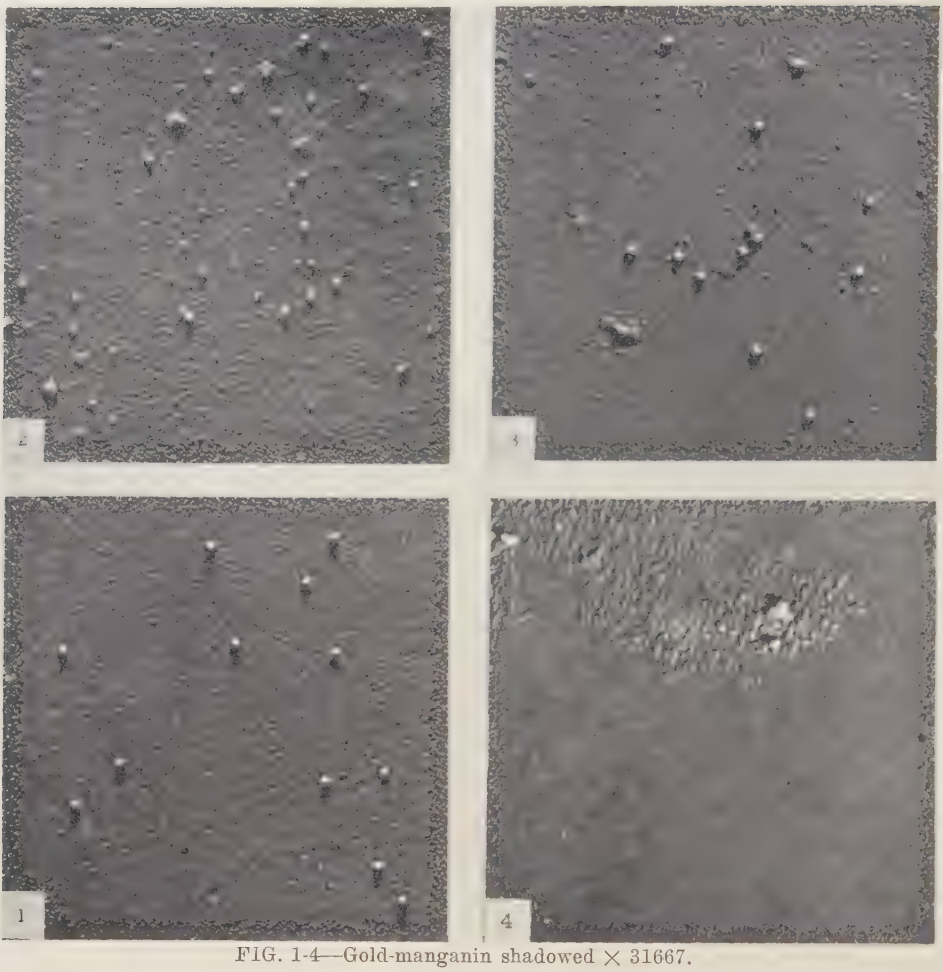

FIG. 1-4—Gold-manganin shadowed × 31667.

FIG. 1. Particles in tissue culture fluid of Type 1 poliomyelitis virus (Mahoney).
FIG. 2. Particles in tissue culture fluid of Type 2 poliomyelitis virus (Y-SK).
FIG. 3. Particles in tissue culture fluid of Type 3 poliomyelitis virus (Leon).
FIG. 4. Amorphous material found in some screens prepared from control uninoculated, but incubated, tissue cultures.

hour. The supernatant liquid was now discarded and, after allowing the inverted tubes to drain dry on gauze, they were carefully examined but no pellet of any kind was discernible. The tubes were rinsed with 5 ml of Seitz-filtered distilled H_2O, using a round-end capillary pipette forcibly to spray the sides and base of the tube. At the same time the tip of the pipette was gently rubbed along the surface of the tube to dislodge the invisible sediment. This "wash-fluid" was then diluted to 12 ml with water and centrifuged at 6,600 g for 30 minutes in steel tubes in a Spinco No. 40 rotor to remove any aggregates. The resulting supernatant liquid was then transferred to other tubes which were spun at 92,000 g for one hour. After discarding this supernatant liquid, 0.5 ml of water was used to wash each tube as before. This was water-clear, devoid of gross particles, and represented a 222-fold concentration of the original fluid. The final concentrates were not titered but, if one assumes that little or no virus was lost during this manipulation, the Type 1 material would have had $10^{10.2}$ TCD_{50} per ml, the Type 2 and Type 3—$10^{8.5}$ TCD_{50} per ml. The concentrates were placed on collodion-coated screens, shadowed with gold-

TABLE III. Size of Particles in Electron Microscope Photographs of Concentrates from Tissue Culture Fluids Containing 3 Types of Poliomyelitis Virus.

Type of poliomyelitis virus	No. of particles measured	Range of size, mμ	Mean size, mμ
1 (Mahoney)	117	28.9-33.3	30.3
2 (Y-SK)	103	23.2-29.6	26.9
3 (Leon)	115	37.1-43.5	41.2

manganin at arc tan 1/5 and observed with the RCA, EMU electron microscope. The electron micrographs were prepared by Mr. Lucien Caro.

Fig. 1-3 illustrate typical fields of the preparations from the virus-containing tissue culture fluids. Screens of the control, uninfected tissue culture fluid concentrate were generally free of significant debris; the appearance of occasional areas of amorphous material is shown in Fig. 4. The diameters of these particles as they appear on the negatives (not the prints) were measured with a precision measuring engine and the results are shown in Table III. The difference between the mean size of 27 mμ of the Type 2 particles and 30 mμ of the Type 1 particles is probably not significant. It is noteworthy that, after this work was completed, Stanley, Bachrach, and Schwerdt(5) reported finding particles having a diameter of 28 mμ in preparations from 2 other Type 2 strains of poliomyelitis virus, Lansing and MEF_1, and that the property of infectivity resides solely in the 28 mμ particle. Since no other significant particles were found in our tissue culture preparations, it is highly probable that those presented and measured in the accompanying photographs represent the virus. The somewhat smaller size of the poliomyelitis viruses obtained by gradocol membrane filtration using Elford's formula may have several explanations. It may be that the particles spread out and appear larger when they dry on the collodion film or it may mean that Elford's formula needs revision. In this respect it should be noted that both Leyon and Gard(6) and Melnick, Rhian, Warren, and Breese(3), on the basis of sedimentation data, assigned a size of 28 mμ to the Lansing strain of Type 2 poliomyelitis virus.

One still needs to consider, however, the marked discrepancy in the size of the Type 3 particles, mean of 41 mμ, particularly when the gradocol membrane filtration data were similar for all 3 types. While there is no obvious explanation for this discrepancy, it is perhaps noteworthy that the Type 3 cultures were found to have a "mucinous" or other "sticky" substance which quickly glazed and clogged the gradocol membranes. If this substance, either a part of the virus particles or adsorbed on them, were responsible for more extensive gold-manganin shadowing, it might explain the seemingly larger size of the Type 3 particles in the photographs obtained with the electron microscope. If one compares only the white spheroids, the Type 3 and Type 1 particles are more nearly alike in size.

Summary. When the concentration of virus in stock filtrates of the tissue culture fluids was greater than 10^6 TCD_{50} per ml, membranes with an APD of 39 mμ to 44 mμ allowed a certain proportion of poliomyelitis virus particles to pass. Membranes with an APD of 36 to 39 mμ held back the virus and, according to Elford's formula, the size of all 3 types of poliomyelitis virus is estimated at 12-20 mμ. After removal of gross amorphous material by preliminary centrifugation, the tissue culture fluids yielded practically pure suspensions of virus particles which measured, on the average, 30 mμ for Type 1 (Mahoney), 27 mμ for Type 2 (Y-SK), and 41 mμ for Type 3 (Leon). The seemingly larger size of the Type 3 particles may be an artifact.

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Metabolism of Choline and Related Compounds by Hepatic Tissue From Several Species Including Man.* (20882)

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The enzymatic activity of the same tissue or organ is known to vary widely from species to species. Differences in choline oxidase activity of hepatic tissue are particularly marked(1,2). It is the purpose of the present report to record the variation among species of the activity of the enzyme systems involved in the oxidation of choline and succinate and the formation of methionine employing choline or betaine or dimethylthetin as methyl donors and homocysteine as the methyl acceptor. Data on the activity of fresh human liver are included. This study represents one phase of a more general study of the possible relationship between the production of liver tumors in rats by chronic choline deficiency and their production by the carcinogenic azo dyes(3).

Methods. Succinoxidase and choline oxidase activity were measured manometrically using the methods reported by Potter and Schneider(4) and Kensler and Langemann(5) respectively. Methionine formation was measured colorimetrically using the method of McCarthy and Sullivan(6) as modified by Dubnoff and Borsook(7). Methionine synthesis when choline or betaine were employed as methyl donors was carried out under conditions essentially the same as those used by Dubnoff(2) except for higher concentrations of choline (0.01 M) and betaine (0.02 M). The incubation period was one hour. Transmethylation from dimethylthetin was measured using 10 mg of tissue, a pre-incubation period of 60 minutes at 38°C, and a reaction time of 5 minutes, conditions which were found earlier(8) to give maximal values. Tributyrinase activity was measured manometrically using 1 mg (wet weight) or less of tissue per manometer. For ease of comparison among the different enzymatic activities measured, they are all expressed as Q values, *i.e.* mm^3 of gas consumed or produced (volumetric equivalent of the amount of methionine formed) per mg dry weight of tissue per hour. As dry weight-wet weight ratios were not determined for all species or samples, it has been arbitrarily assumed that the dry weight equals 30% in all cases. The samples of human liver were obtained by biopsy during abdominal operation, immediately immersed in ice cold Ringer-phosphate solution and then homogenized in glass homogenizers and the assays started within 30 minutes of removal. Microscopic sections were obtained of the liver in two cases and were morphologically normal. All 4 were cholecystectomy cases. We are indebted to Dr. T. H. Jukes of the Lederle Laboratories for the 4- to 6-week-old New Hampshire Red (NH) and hybrid (H) chicks used in this study. They were maintained on a casein base ration and were the completely supplemented controls used in a study of vit. B_{12} and folic acid deficiency(9). The rats and mice were on a dog pellet ration and the rabbits and guinea pigs received rabbit pellets (Rockland) and greens.

Results. The data obtained are summarized in Table I. Choline oxidase activity, in agreement with earlier reports(1,2) varies widely from species to species. Adult rat liver contains the greatest activity whereas guinea pig, rabbit and human liver contained little or no detectable activity. The low values reported for rabbit and human liver fall within the experimental error of the method. In agreement with Dubnoff(2) no methionine formation from choline and homocysteine was observed with guinea pig liver. Rabbit and

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TABLE I. Enzymatic Activity of Liver from Various Species.

Species	Strain or wt group	Choline oxidase, Qo_2	Succin-oxidase, Qo_2	Transmethylation from— Choline, Q meth	Betaine, Q meth	Dimethyl-thetin, Q meth
Rat	6–49 g	2 (16)*	65 (10)*	.04(10)*	.43(10)*	63(15)*
	50–85 g	3.2 (9)	78 (9)	.06(9)	.38(9)	68(8)
	85 g	6.5 (24)	78 (10)	.09(20)	.32(20)	69(19)
Mouse	C_3H	2.0 (4)	90 (4)	.20(4)	.60(4)	120(6)
	A	2.0 (5)	93 (5)	—	—	97(5)
	C	1.7 (3)	77 (3)	.11(2)	.52(3)	72(3)
Chicken	NH	1.9 (10)	50 (10)	.10(10)	.24(10)	10(10)
	H	2.5 (10)	55 (10)	.16(10)	.32(10)	19(10)
Dog		1.9 (2)	63 (2)	—	—	28(2)
Rabbit		.10(5)	37 (5)	0 (5)	.17(5)	30(5)
Man		.06(3)	5.9(4)	0 (3)	.26(3)	16(4)
Guinea pig		.0 (4)	54 (4)	0 (4)	.39(4)	32(4)

* No. of animals assayed.

human liver also produced no detectable methionine when choline was the methyl donor. Mouse, rat and chicken livers were active.

In contrast to the results obtained with choline, when betaine was the methyl donor methionine was synthesized by the livers of all species examined. Dimethylthetin (the sulfur analog of betaine) was much more effective than betaine as a methyl donor to form methionine from homocysteine in the livers from all species examined. It will be noted, however, that the relative effectiveness of dimethylthetin and betaine varies from species to species. This may indicate that two different enzymes are involved or that substrate affinity (specificity) of a single enzyme varies from species to species. Dubnoff and Borsook(7) concluded that 2 different enzymes were involved in rat liver on the basis of a selective loss of betaine transmethylase activity from tissue extracts transmethylating from both betaine and dimethylthetin. In their experiments, activity was measured under conditions which were nearly optimal for betaine but suboptimal for dimethylthetin (Q—1.5 as opposed to 69 in present study). Thus, a decrease in betaine activity would be readily detected whereas a similar decrease in dimethylthetin activity might be missed.

As is indicated in Table I, only 4 samples of human liver were obtained for analysis. All were from females between 35 and 59 years of age who had been under ether anesthesia for from 1 to 3.5 hours prior to the excision of the liver tissue. No obvious differences in enzymatic activity with respect to age or duration of anesthesia were noted. The Q value for choline oxidase varied from 0 to .1, that for succinoxidase from 2.1 to 10., that for methionine formation from betaine from .13 to .33, that for methionine formation from dimethylthetin from 11 to 20. No methionine formation was detected in any of the 3 livers tested with choline as the methyl donors. The succinoxidase activity values are surprisingly low but are in agreement with the low values reported by Waterlow(10) for succinoxidase and cytochrome oxidase activity in human liver. Tributyrinase activity was measured in 2 cases (Q = 1520, 1930) and found to be of the same order of magnitude as that found in the liver of rats and mice (Sherman strain rats Q = 1120: C_3H mice Q = 3235).

Discussion. On the basis of the limited data so far obtained on adult human liver tissue, it appears that enzymes are present which actively methylate homocysteine to form methionine when either betaine or dimethylthetin are the methyl donors. No methylation of homocysteine could be detected when choline was the methyl donor. This pattern of activity is thus similar to that observed in liver tissue from rabbits and guinea pigs. Choline oxidase activity is also low or

absent in these three species in contrast to the at least 15- to 60-fold higher levels observed in the other 5 species examined.

The bulk of the work that has been done on choline requirements and metabolism has been done on rats, mice and dogs(11,12). Comparatively little work has been done with guinea pigs or rabbits. Handler(13) has fed seven different choline deficient diets to guinea pigs and rats. All seven led to a marked increase in liver lipids in rats but only the peanut meal base ration which increased rat liver lipids to as high as 25% of the wet weight caused a slight increase in guinea pig liver lipid content. Choline administration was without effect on this increase in liver lipids in the guinea pig. Dubnoff(14) has reported that C^{14} methyl labelled groups of choline are found in the methyl groups of tissue proteins. Whether this represents transmethylation or oxidative demethylation and resynthesis to methyl groups has not been established. In the rabbit, Spellberg, Keeton and Ginsberg(15) reported the dietary production of cirrhosis which was not prevented by vit. B concentrates containing choline although Blumberg, MacKenzie and Seligson (16) have produced cirrhosis in young rabbits and prevented its development with large amounts of choline. Transmethylation from methionine to form choline has been found to occur in rabbits(17). In man, Simmonds and du Vigneaud(18) have shown that the methyl group of methionine is utilized to form choline and creatinine. No comparable studies have been made on the lability of the methyl group of choline in man or the rabbit. In view of our preliminary findings in regard to the *in vitro* failure to detect transmethylation from choline to homocysteine in human as well as guinea pig and rabbit liver, further study of this process in these species appears desirable.

The data confirm and extend the observations of Dubnoff(2) that transmethylation from choline to homocysteine is detectable *in vitro* only in those species which possess an active choline oxidase system. In the rat where a sufficient number of animals in different age groups have been examined, it appears that transmethylation from choline, as well as choline oxidase activity(19), increases with the growth of the animal. Transmethylase activity from betaine or dimethylthetin in the livers of the same animals does not show this change.

Summary. 1. Data on the activity of choline oxidase, succinoxidase, tributyrinase and the enzymes concerned in the formation of methionine from choline, betaine or dimethylthetin and homocysteine are presented for liver tissue from the rat, mouse, guinea pig, rabbit, chicken, dog and man. 2. Analysis of 3 samples of fresh human liver indicate that man resembles rabbit and guinea pig in that choline oxidase activity and capacity to utilize choline to methylate homocysteine are low or absent. 3. Livers of all species examined were capable of utilizing betaine and its sulfur analog dimethylthetin to methylate homocysteine to form methionine. 4. Succinoxidase activity of 4 samples of fresh human liver were markedly lower than that of other species examined. Tributyrinase activity of two samples examined was of the same order of magnitude as that found in livers from the rat and the mouse.

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Mouse-Strain Differences in Response to Radiation. (20883)

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In the course of an experiment(1) on the protective effect of hormones on the mortality of irradiated mice, there was observed a considerable difference in the radiation doses delivered to the heads of mice of 2 different strains necessary to elicit the same degree of mortality. The doses differed by more than a factor of 2 in the dba and Marsh-albino mice.

The difference in acute radiation mortality has now been examined in 2 additional mouse strains, making a total of 4. This is a report on the results to date, which were obtained with each strain represented by several hundred mice of either sex in the age range from 8 to 15 weeks. The entire experiment is being repeated with larger numbers of mice as well as with additional mouse strains.

Procedure. In order to establish the acute mortality curves, mice of the dba, Marsh, C57 black, and C3H strains were irradiated as follows: (a) whole body exposed, (b) only the head irradiated, and (c) the head shielded and the remainder of the body irradiated. All mice were irradiated at a distance of 30 cm to the midsection with an x-ray beam having a H.V.L. of 0.9-mm copper and a dose rate of 150 r per minute in air. The x-ray generator was monitored continuously for constancy of output. Doses reported herein are air doses. For whole-body radiation 10 mice were placed in a 6″-diameter Petri dish and covered with a perforated aluminum disc ½-mm thick. Since the space was limited, it was not necessary to immobilize the animals by anesthesia. Measurements of dosage when the dish was filled with purple-heart wood to simulate the setup when filled with mice showed that the radiation dose intensity decreased from 100% at the center to 91.8% at the outer periphery of the dish. Thus, the doses to the mice varied approximately 4% above and below the average dose in the dish. For irradiation of the head only the mice were held in position by a half cylinder of 1/16″ lead which functioned both as an immobilizing device and as a radiation shield. Only the head was exposed. The mice and shield were fastened to a wooden board in 2 rows of 3 mice each, nose to nose, so that the heads of 6 mice could be irradiated simultaneously. Over the bodies of the mice covered with 1/16″ lead 2 additional layers of 1/8″ lead rubber were laid. Transmission of the x-rays through one layer of 1/8″ lead rubber is 0.06%. The boundary between the irradiated head and shielded body was defined by the edge of the lead-rubber shield, which ran across the cervical vertebrae. Measurements of radiation dose in this setup were made with the mice simulated by purple-heart wood mock-ups. At the center of the mouse body under the shield the dose due to scattered x-rays was 4% of the dose delivered to the exposed head. For body irradiation without head the above procedure was modified by using a semicylinder of ½-mm-thick aluminum instead of lead to restrain the mice. The heads were shielded by 1/8″ lead. The edge of the lead defined the boundary between head and body and ran across the cervical vertebrae. Dosage measurements with the purple-heart wood mock-ups showed that the

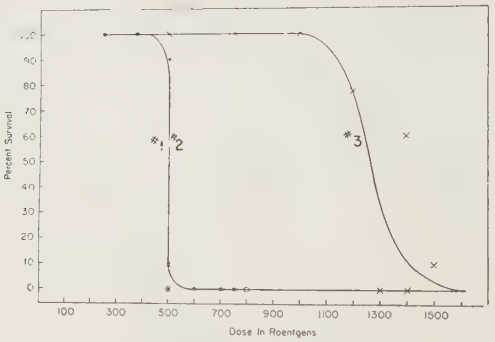

FIG. 1. Survival curves for the DBA strain. ● #1 whole body exposure; ○ #2 head exposure; × #3 body exposure, head protected.

dose in the center of the shielded head due to scattered x-rays was 13% of the dose delivered to the exposed body.

Results. The survival rates plotted against radiation doses for 4 strains of mice under the 3 conditions stated above are shown in Fig. 1, 2, 3, and 4. These curves show there is a marked strain difference in the response of these mice to the 3 different conditions of radiation. When the whole body is irradiated, the resulting survival curves for the 4 strains are practically identical, having a median lethal dose (M.L.D.) at about 528 r. The survival was 50% at 12 days. Thus, 528 r is the L.D.-50% at 12 days.

From the curves for the dba strain (Fig. 1) it is seen that the survival curve with head radiation is practically identical with that for whole-body radiation whereas, when the head was protected, over double the dosage was required to produce the same effect. This would seem to indicate that in this dba strain a radiosensitive center is located in the head region.

On the other hand, the curves for the Marsh strain (Fig. 2) show that the survival curve for the head radiation lies in a higher dosage range, at about double the dosage for whole-body exposure, whereas the curve for whole-body-with-head-shielded radiation has moved to a lower dosage range than the corresponding curve for the dba strain.

The survival curves for the C57 black mice (Fig. 3) present still another picture in that there is a more marked separation resulting from the 3 methods of radiation. In this strain the survival curve following head radiation falls at a higher dosage level, and the curve for the whole body with head shielded falls at a lower level than the corresponding curves for the Marsh and dba strains. These

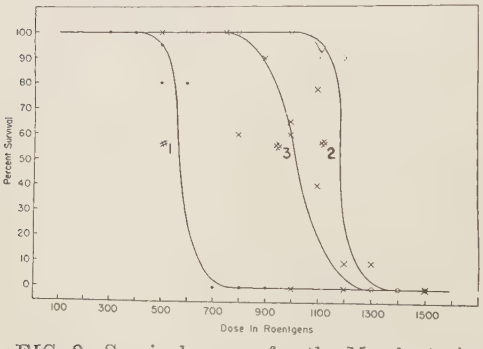

FIG. 2. Survival curves for the Marsh strain. ● #1 whole body exposure; ○ #2 head exposure; × #3 body exposure, head protected.

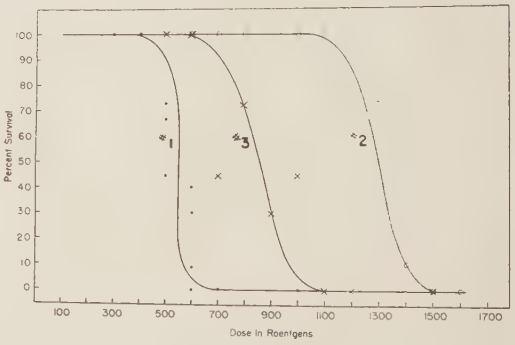

FIG. 3. Survival curves for the C57 Black strain. ● #1 whole body exposure; ○ #2 head exposure; × #3 body exposure, head protected.

findings could be explained if the head region were less sensitive to radiation than the remainder of the body. The curves for the C3H strain show this difference to a more marked degree in that the head-radiation curve is at a still higher dosage level, whereas the curve plotted for the mice radiated whole body with head shielded is at a lower level, suggesting that the head region in this strain is appreciably less sensitive to radiation than that in any of the other 3 strains.

With the families of curves arranged in the order shown, it should be noted that, as the head-radiation curves move progressively toward the higher dosage levels, the curves for the whole-body-except-head radiation move progressively toward the lower dosage levels.

TABLE I. M.L.D. Values for the Three Conditions of Irradiation.

Mouse strain	Whole body, r	Head, r	Body except head, r
Dba	500	500	1265
Marsh	570	1185	1018
C57 black	550	1300	858
C3H	492	1443	735
Avg	528		

This is shown more clearly in Table I where the M.L.D. is given for comparison. From Table I it may be inferred that, as the head region becomes less sensitive to radiation, the remainder of the body becomes more radiosensitive.

The mortality curves shown in Fig. 1, 2, 3, and 4 refer to an acute mortality. Thus, the survival times for 50% of the mice given whole-body irradiation were in the interval of 10 to 12 days. When the head alone was irradiated, 50% survived for periods of from 12 to 20 days; and in the case of body-with-head-protected irradiation the survival was 50% for times ranging from 15 to 25 days.

The animals were examined daily for body changes. The most obvious symptoms of injury were an arched back, bristling fur, reduction of activity, and loss of weight. No evidence of infection was found. In those animals that survived for a sufficiently long time, hair and eye changes were seen. The C57 black and C3H mice which survived 1400 r to the head showed epilation of head hair from the 19th to the 21st days. About 2 weeks later the epilation was replaced by a growth of gray hair. This gray hair showed the sharply defined boundary between head and body across the cervical vertebra. At the lower dose of 1100 r partial epilation was seen. The hair did not fall off spontaneously, but could be rubbed off easily.

Eye changes were readily discernible with doses of 1400 and 1500 r delivered to the head. Inflammation of the epithelia surrounding the orbit preceded the appearance of visible cataracts at 3 weeks after radiation.

Discussion. The progressive changes in the values of M.L.D. with decreasing sensitivity of the head region of the mouse are greater than the data shown in Table I would indicate. The dosage measurements on mock-up mice indicated that in the whole-body-without-head exposures there was a scattered dose given to the shielded head, which was 13% of the body dose. Therefore, the M.L.D. values under the column for whole-body-except-head irradiation are probably lower than they would be if it were possible to irradiate the mouse body without scattering dosage into the head region.

The probable contribution of the scattered dose into the head is illustrated in the dba mice where 13% of the 1265 r (M.L.D. for whole body without head) is 164 r which, in turn, is 33% of the M.L.D. of 500 r for head only. In the C3H mice 13% of 735 r (M.L.D. for whole body without head) is 96 r. This, however, is only about 7% of the M.L.D. of 1443 r for head only. Thus, the scatter of radiation has the effect of decreasing the observable strain differences in mortality. In the C3H mice the scattered dose is probably negligible, whereas in the dba mice it assumes a significant role in the acute mortality observed.

The wide range of sensitivity of the head region shown by the M.L.D. values of Table I are not easily accounted for by the assumption of a single radiosensitive center in the head, namely the pituitary. The ratio of M.L.D. for irradiation of head only to that for whole-body-without-head irradiation is 0.40 for dba, 1.1 for Marsh, 1.5 for C57 black, and 1.96 for C3H mice. Measured by this ratio, there is more than a 4-fold change of sensitivity of the head compared with the remainder of the body. This estimate does not take

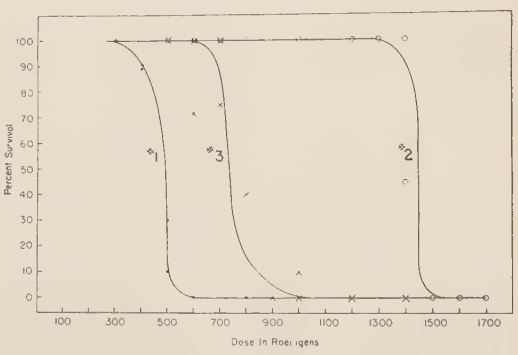

FIG. 4. Survival curves for C3H strain.
● #1 whole body exposure; ○ #2 head exposure; × #3 body exposure, head protected.

into account the scattered dose to the head which was discussed above.

Two instances of different degrees of radiosensitivity of the head have been reported in other animals. Allen(2) reported that a lead shield over the heads of mongrel dogs, permitting not more than a 50-r dose to the head, enabled 90% of the dogs to survive a 450-r whole-body dose. This dose, given without head shield, caused 100% mortality. On the other hand, Lamerton *et al.*(3) found that doses of 600 and 1500 r to the head of the Wistar-albino rat produced little effect on the growth curve of young rats. They concluded that in these rats pituitary damage was not an important factor in subsequent rat growth. They report, however, in a comparison between rats given 450-r whole-body-with-head exposure and others given 500-r without-head exposure that the head-shielded rats showed the protective effect against radiation.

The dba mice have the same high degree of radiosensitivity in the head region as that reported by Allen for mongrel dogs. The other mouse strains show less sensitivity. The C3H mice appear to have the low head sensitivity of the Wistar rats, as reported by Lamerton.

That the sensitive center in the head might be the pituitary was indicated by the protective action of the adrenosteroid hormones against radiation mortality, as reported by Mirand *et al.*(1). Both cortisone and desoxycorticosterone acetate were found to reduce radiation mortality by their effects on the pituitary-adrenal axis. The assumption was made that the extent of pituitary damage as a result of ionizing radiation determines the degree of adrenal insufficiency which might be the large factor contributing to the death of the animals. The fact that both DCA and cortisone increased the survival time in the dba mice suggests that a state of adrenal insufficiency was avoided either by rendering the pituitary less sensitive to ionizing radiation or by preventing an early marked manifestation in adrenocortical insufficiency after radiation.

Summary. 1. The acute radiation mortality of mice exposed to x-rays shows that there are radiosensitive regions in the mouse head. 2. The L.D.-50% at 12 days for acute mortality due to whole-body exposure to x-rays is essentially the same for 4 mouse strains and is about 528 r. 3. The sensitivity of the head region varies with mouse strain. In the most sensitive strain, the dba, the M.L.D. for head exposure is the same as for whole-body exposure. Up to more than a 4-fold decrease in head sensitivity in the strains is found in the following order: dba, Marsh, C57 black, and C3H mice.

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Antagonism of Cortisone to Body Temperature Reducing Effect of Histamine.* (20884)

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Mice inoculated with *Hemophilus pertussis* vaccine are more sensitive to the lethal effects of histamine, of anaphylactic shock, of heterologous vaccines, and, of anoxia than uninoculated animals(1,2). Cortisone inhibits histamine death in pertussis-injected mice(3). It is believed that further work on the mechanism of cortisone-histamine antagonism will be facilitated by the use of a less complex endpoint than death. Fabinyi and Szebehelyi(4) have shown that histamine decreases the body temperature of mice at 18-20°C. The following experiments will demonstrate that pertussis-inoculated mice are more sensitive to the temperature-lowering effects of histamine than uninoculated animals, and, that cortisone will reduce the fall in rectal temperature of both pertussis-inoculated and uninoculated animals. It is hoped that this study will focus attention on the histamine-antagonizing ability of cortisone, since this property may be of some importance in the anti-allergic activities of this hormone.

Materials and methods. White, 17 to 25 g mice, bred in our laboratory from a strain originally purchased from Tumblebrook Farms, were employed. Since female mice have been reported to be more sensitive to histamine than males(5), either all females or all males were used in any one experiment. A group of female mice was injected with 0.2 ml of *H. pertussis* vaccine† containing 47.7 billion organisms per ml. Five days later, both pertussis-inoculated and uninoculated animals were placed in a room thermostatically controlled to 18°C. Rectal temperatures of all animals were taken by means of a copper-constantan thermocouple connected to a galvanometer. Pertussis-inoculated mice were then divided into 3 groups, each group consisting of 10 animals. The first batch of mice was injected subcutaneously with 5 mg/kg of histamine dihydrochloride; the second and third groups, with 10 mg/kg and 20 mg/kg, respectively. Eighteen uninoculated mice were separated into 3 equal groups, and each group was challenged with subcutaneous doses of either 25, 50, or 100 mg/kg of histamine dihydrochloride. Rectal temperatures of all animals were recorded at ½, 1, 2, and 4 hours after the administration of histamine. Twenty mg/kg of histamine killed 4 of 10 pertussis-inoculated animals, and 100 mg/kg of this amine was lethal to 1 of 6 uninoculated mice. Any temperature readings obtained from animals who subsequently succumbed were discarded. The data in Fig. 1 thus represent the average temperatures of survivors at various time intervals after histamine administration. In a *second experiment,* only male mice were employed. Ten pertussis-inoculated and 10 uninoculated animals were challenged with 25 mg/kg and 400 mg/kg of histamine respectively. These doses of histamine killed 4 of 10 pertussis-inoculated mice and 5 of 10 uninoculated animals. Only temperatures of

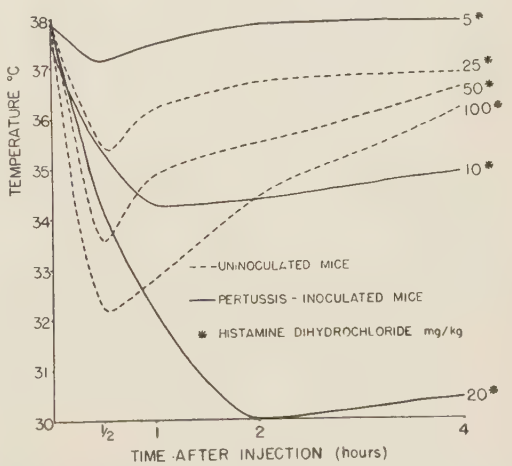

FIG. 1. Body temperatures of uninoculated and pertussis-inoculated mice after administration of varying doses of histamine dihydrochloride.

* This study was supported by a grant from the U. S. Public Health Service.

† Supplied through the courtesy of Dr. J. Munoz of Sharp & Dohme, Inc.

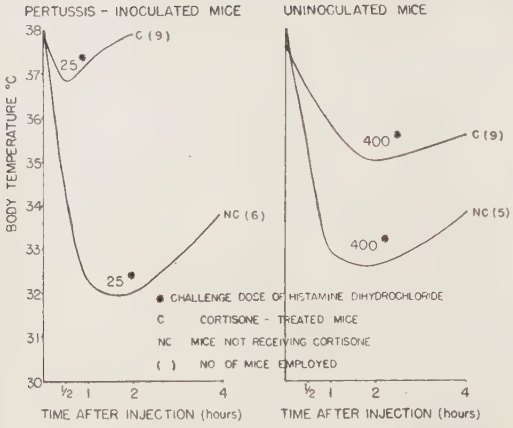

FIG. 2. Antagonism of cortisone to body temperature reducing effect of histamine in pertussis-inoculated and uninoculated mice

survivors were averaged. In addition, 9 pertussis-inoculated and 9 uninoculated animals were injected intramuscularly with 2.5 mg of cortisone acetate (Upjohn) 18 hours before challenge doses of histamine were administered. All pertussis mice inoculated with cortisone survived 25 mg/kg of histamine, and all normal control animals injected with cortisone survived 400 mg/kg of this amine. Temperatures were recorded as described in the previous experiment.

Results. The effect of varying doses of histamine on the body temperature of pertussis-inoculated and uninoculated mice is shown in Fig. 1. It is evident that pertussis-inoculated mice are more sensitive to the temperature-reducing effects of histamine than uninoculated animals. Ten mg/kg of histamine in pertussis-inoculated mice resulted in approximately the same temperature drop as 50 mg/kg in uninoculated animals. Likewise, the data in Fig. 2 demonstrate that 25 mg/kg of histamine resulted in a fall in temperature in pertussis-inoculated mice comparable to that produced by 400 mg/kg in uninoculated animals. The data in Fig. 2 also indicate that cortisone reduces the fall in body temperature after histamine in both pertussis-inoculated and uninoculated mice. The maximum temperature drop in pertussis-inoculated mice without cortisone was 5.9 $\pm$ 1.3°C; in pertussis-inoculated, cortisone-treated mice, 1.0 $\pm$ .82°C. The difference between these values is a significant one, ($p = <.01$). The fall in temperature of uninoculated mice without cortisone was 5.2 $\pm$ 1.2°C; the temperature drop in uninoculated cortisone-treated mice was 2.3 $\pm$ .23°C. The difference between these values is a significant one, ($p = .05$).

Discussion. The body temperature reducing effect of histamine in mice appears to offer a convenient method for studies of cortisone-histamine antagonism. Gyermek believes that histamine lowers body temperature at 20°C by dilating skin capillaries thus increasing heat loss(6). Since cortisone has been reported to augment the vasoconstrictor action of epinephrine(7), its antagonism to histamine in our experiments may be due to a potentiation of the vasoconstrictor effect of endogenous epinephrine on skin vessels with a resultant decrease in heat loss.

Summary. Pertussis-inoculated mice are more sensitive to body temperature lowering effect of histamine than uninoculated animals. Cortisone reduces the temperature drop in both pertussis-inoculated and uninoculated mice.

We are indebted to Mr. Eugene Woods for his technical assistance, to Dr. H. R. Pratt-Thomas for the use of his constant-temperature room, and to Mr. Oliver Brodie for the use of his galvanometer and thermocouple.

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Particle Size of Antigen in Relation to Antibody-Suppressive Effect of Cortisone. (20885)

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From the U. S. Department of Health, Education and Welfare, Public Health Service, National Institutes of Health, National Microbiological Institute, Bethesda, Md.

We have previously reported that in rabbits simultaneously immunized with bovine serum albumin (BSA) and either mumps virus(1) or sheep red cell stroma(2) the administration of cortisone uniformly suppresses production of anti-BSA but affects the production of antibody to mumps virus only irregularly and the production of antibody to stroma not at all. These findings suggested that the ability of cortisone to suppress antibody production in the rabbit was influenced by the particle size of the antigen. To determine whether or not this was so the ideal experiment would involve the study of the rabbit's response to the same antigen in 2 widely different sizes, so that factors other than size could be constant. A near approach to this situation was obtained by immunizing rabbits with either BSA or human red cell stroma conjugated with p-bromophenylisocyanate and measuring the antibody thus produced with yet another bromophenylureido-protein so that in each instance antibody only to the phenylureido moiety was measured.

Materials and methods. Preparation of antigens. Three p-bromophenylureido antigens were prepared. Conjugated bovine serum albumin† (PBSA) and egg albumin‡ (PEA) were prepared by the method of Wormall and Hopkins as given by Kabat and Mayer(3). The conjugated red cell stroma (PRC) were prepared in a similar fashion except that to rid the PRC of the bromodiphenylurea reaction product it was necessary to wash with alcohol-ether and ether since solution in alkali and precipitation by acid could not be used to isolate the antigen in this case. The bromine served as a marker to provide some indication of the number of phenylureido linkages on each antigen. Table I lists the Br, N and moisture determinations on these antigens. Since the method used for Br analysis would be influenced by the presence of I, some unconjugated stroma were washed in the same way as the PRC and also analyzed for Br. None was detected. *Antibody N determinations.* When PBSA and PEA were used Antibody (Ab) N was determined by the quantitative precipitin method(4). Antibody to PRC was determined by the quantitative agglutinin method(5).

TABLE I. Analytical Data for Antigens.

	% N	% Br	% moisture
PBSA	13.7	3.2	9.7
PEA	13.4	2.9	10.4
PRC	10.9	5.7	6.0

Experimental. In the first experiment 10 albino rabbits each weighing approximately 2 kg were immunized with 7 intravenous doses of 2 mg each of PBSA given over a period of 2 weeks. From 3 days before until 6 days after the antigen injections 5 of these rabbits were given 4 mg of cortisone§ daily intramuscularly. Six days after the last Ag injection all the rabbits were bled and the serum separated. There was one death in each group (control and cortisone) during immunization.

Results. Quantitative precipitin curves were set up with each of the sera against PBSA and PEA and the Ab N levels thus determined are recorded in Table II. It will be seen that the control group averaged 2.3 (anti-PBSA) or 2.4 (anti-PEA) times as much Ab N as did the cortisone treated group and that moreover there is a distinct separation of the ranges of the 2 groups. Thus the results obtained in rabbits immunized with PBSA are the same as for BSA(1,2). The

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† Crystallized Bovine Plasma Albumin, Armour and Co., control No. 127-250.

‡ Crystallized Egg Albumin, Armour and Co., lot No. E 90115.

§ Cortone, Merck & Co.

TABLE II. Antibody N Levels in Rabbits Immunized with PBSA and Treated or Not Treated with Cortisone.

Group	Animal #	Anti-PBSA, γAb N/ml	Anti-PEA, γAb N/ml	Anti-PSA / Anti-PEA
Cortisone	145	43	14	3.1
	146	22	5	4.4
	147	70	29	2.4
	148	67	19	3.5
Avg		58	17	3.4
Controls	150	95	29	3.3
	151	180	61	3.0
	152	122	34	3.6
	154	142	40	3.6
Avg		135	41	3.4

homologous Ag, PBSA, precipitated an average of 3.4 times as much Ab N as did a heterologous conjugated Ag, PEA. That this difference was not due to Ab to native BSA was shown by the fact that when quantitative precipitin curves with these sera were set up with BSA very small amounts of precipitate were obtained with sera No. 151, No. 152, and No. 154 and none with the other sera. The difference presumably is due to differences in the portion of the antigen molecules partaking in the phenylureido linkage.

In the second experiment 12 albino rabbits were immunized with 7 intravenous doses of PRC and 6 of these were treated with cortisone in the same way as the rabbits of the first experiment. All animals were again bled 6 days after the last Ag injection. Quantitative precipitin curves were set up with each of the sera against PBSA and PEA and one quantitative agglutinin absorption was done with PRC. The results are given in Table III. It can be seen that PBSA again precipitated more Ab than did PEA although in this instance they were both heterologous antigens. However the difference is considerably less than in the previous experiment. The control group averaged a slightly higher level of anti-PBSA and anti-PEA (1.3 and 1.1 times respectively) than did the cortisone-treated group but the Ab N ranges of the 2 groups are the same. In the case of the anti-PRC determination, since only one absorption was done and the values obtained represent small differences between relatively large values, little quantitative importance can be ascribed to these data except that they show that there was anti-PRC. There was however no visible agglutination of the PRC when added to these sera, the PRC resuspending quite as easily in these tubes as in the tubes in which saline was present instead of serum. Good agreement was obtained between the N values of the washed sediment of the PRC-saline tubes and the N content of the stock PRC suspension pipetted directly in the digestion flask (139 μg N for the former and 141 μg N for the latter—average of duplicate analyses) indicating that little soluble protein was leached from the PRC by washing.

Discussion. The results provide evidence that the antibody response to antigen of large particle size is less influenced by cortisone treatment than is the case with smaller, soluble antigens. Why this should be is not too clear but it certainly suggests that the effect of cortisone is not anything so fundamental as an interference with gamma globulin synthesis. On the contrary it suggests that cortisone exerts its effect at some point before the antigen reaches the site of antibody synthesis. It is possible that due to their differing physical states the PBSA and PRC are captured by 2 different elements of the reticulo-endothelial system, and that one of these elements is affected by cortisone whereas the other is not.

There is another line of evidence that also indicates that interference with gamma globulin synthesis is not responsible for the cortisone effect. In our work we have found that whether antibody to BSA in the control group is of the order of 0.5-1.0 mg or 0.05-0.1 mg Ab N per ml serum the average Ab levels in these groups were consistently 2 to 3 times those in the corresponding cortisone-treated group. One would expect that if gamma globulin synthesis was involved that less or no relative suppression would occur when the Ab level in the control was low, because then the rate of globulin synthesis should be more nearly equal to the stimulus for antibody production.

Summary. Rabbits were immunized with either p-bromophenylureido-BSA or similarly conjugated human red cell stroma. One-half the rabbits in each group were treated with

TABLE III. Antibody N Levels in Rabbits Immunized with P-HRC and Treated or Not Treated with Cortisone.

Group	Animal #	Anti-PBSA γ Ab N/ml	Anti-PEA γ Ab N/ml	Anti-PRC*	Anti-PBSA / Anti-PEA
Cortisone	167	40	27	18	1.5
	168	42	31	lost	1.4
	169	80	55	31	1.5
	170	16	10	17	1.6
	171	4	0	17	
	172	10	3	20	
	Avg	32	21	21	1.5
Control	173	11	7	14	1.6
	174	12	lost	9	
	175	78	48	27	1.6
	182	38	23	18	1.7
	183	81	44	28	1.8
	184	30	23	12	1.3
	Avg	42	27	18	1.6

* γ Ab N obtained with one absorption of 1 ml of serum.

cortisone. Antibody was measured with a p-bromophenylureido-protein other than the one used for immunization. Cortisone reduced antibody production to the conjugated BSA but affected antibody produced to the conjugated stroma but little, if at all.

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Stimulatory Effect of BCG on Isoantibody Production in the Rabbit.* (20886)

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Although considerable work has been reported on the study of isoantibodies in the rabbit(1-7), there is little reference to the difficulty in inducing isoantibody production. In our study of the mode of inheritance of blood group antigens in the rabbit, the probability of isoantibody being produced in a particular recipient has been very small, even when tests indicate that differences exist between the cellular antigens of the donor and the recipient. Preliminary observations made while using the test of native resistance to tuberculosis devised by Lurie *et al.*(8) suggested that BCG injection might increase the probability of isoantibody production. There is considerable evidence to support the view that in rabbits and guinea pigs infection with tubercle bacilli leads to a nonspecific increase in the ability of these animals to produce antibody. Lewis and Loomis(9) found that hemolytic antibody titre could be increased more than tenfold following the injection of sheep erythrocytes into tuberculous animals. Lurie (10) attributes this increased productivity to the generally increased activity of the reticulo-

* This study was supported in part by grants from The American Trudeau Society of the National Tuberculosis Association and the National Microbiological Institute of the National Institutes of Health.

endothelial system in animals exposed to tubercle bacilli or their products.

It was with the purpose of testing the effects of the intradermal inoculation of BCG on the ability of rabbits to produce isoantibody that this study was carried out.

Materials and methods. Rabbits of various races and strains which are maintained at this colony were used as donors and recipients. No regard was paid to weight or sex of the animals; the age in all cases was from early maturity to one year. In the first series of immunizations the donors and recipients were chosen at random, since we did not have any typing serum available. In later series, immunizations were based on known antigenic differences so that the recipient did not possess one or more antigens known to be present on the donor's cells. The recipients received 2 ml of donor's whole blood by the intravenous route 3 times a week for 3 or more weeks. The recipients were bled, and the serum obtained was inactivated at 56°C for ½ hour. The presence of antibody was measured by the agglutination test, which was carried out as follows. Using capillary pipettes which dropped approximately .025 ml per drop, 2 drops of the serum dilution were mixed with 2 drops of 2% washed red cell suspension and allowed to incubate in a 37°C water bath for 3 hours. At the end of this time the tubes were centrifuged at 1000 RPM for 2 minutes and then read with the aid of a concave mirror. Reaction was measured as 4+, 3+, 2+, +, or 0, depending upon the degree of clumping. All sera were tested against a panel of rabbit bloods and those which did not show agglutinating ability were further examined for the presence of incomplete antibody by a modification of the Coombs test in which anti-rabbit globulin made in the guinea pig was used. In only one case was incomplete antibody found. The BCG suspension was obtained at first from the Henry Phipps Institute. Later the BCG was grown in our laboratory on potato and glycerol water and then transferred to Sauton's medium. After one week's growth on this medium the surface growth was harvested and dispersed by rotation with glass beads. The suspension was centrifuged at 2000 RPM for 5 minutes and the supernatant diluted so that 85% of light of 540 Å was transmitted through the suspension. Injections of 0.2 ml of this vaccine into the rabbit by the intradermal route gave rise to a lesion that reached its maximum size in about 3 weeks and then regressed until, after about 8-12 weeks, it completely healed. The first injection of the rabbit cells followed the BCG injection by 5 days.

TABLE I. Attempts at Isoimmunization.

Series	No. of animals	No. producing antibody	P*
1	12	1	No P
2	7	4	BCG P
3a	5	1	" P
3b	6	0	No P
4a	6	0	BCG P†
4b	8	6	BCG P
5	12	9	" P
6a	8	6	" P
6b	9	2	No P‡

* P = Pretreatment.
† Viability of injected organisms doubtful.
‡ Non-producers in this group were later treated with BCG, and immunization was continued. Four of these 7 produced antibody.

Results. Table I shows the results of 6 series of immunizations involved in a test of the BCG technic. Of the series shown, No. 6 was a deliberate attempt to test the efficacy of the BCG injection as a stimulus to antibody production. All the other series were incidental to the study of resistance to tuberculosis, and the groupings into series were based upon factors which are involved in that study and which do not affect isoimmunization. Since handling and treatment of the animals were identical in all series and since statistical analysis indicates homogeneity of these series, it is felt that, for the final analysis, the various series can be treated as one homogeneous group.

In series No. 6, 17 animals were chosen at random from the population and divided into 2 groups. Group 6a, consisting of 8 animals, received a preliminary BCG injection, followed by the standard injection of rabbit red blood cells; Group 6b, consisting of 9 animals, received the standard injection of rabbit erythrocytes without the BCG pretreatment. By the end of the period in which a

total of 12 injections of cells was carried out, 6 of the 8 BCG-treated animals showed antibody production, while only 2 of the 9 which received no pretreatment showed evidence of antibody production. One week later the 7 animals in No. 6b which had not produced antibody were injected with BCG. Three weeks later, after 6 more injections of red blood cells, 4 of these 7 produced isoantibody. A total of 10 out of 15 animals produced antibody after BCG pretreatment as compared with 2 out of 9 without pretreatment.

In series No. 4a the BCG administered to the animals being immunized was also used for a test of resistance to tuberculosis by the Lurie method. On the whole this test was a failure, since the lesions which developed were very small and regressed rapidly. Furthermore, a sample of the vaccine spread on Lowenstein's medium for a count of viable organisms gave negative results at a 1/1000 dilution. It can be seen that none of the 6 animals in this series produced isoantibody. At the same time series No. 4b was started with BCG vaccine which was prepared as a different batch in our laboratory. It can be seen that here 6 of 8 animals produced antibody.

Altogether 3 out of 27 rabbits produced isoantibody with no BCG pretreatment and 26 out of 46 rabbits produced isoantibody following BCG injection. Statistical tests using the χ^2 method indicate that these results are significant at the .01 level.

Discussion. The results indicate quite clearly that the pretreatment with BCG greatly increases the probability of isohemagglutinin being produced. The role of BCG in relation to specificity must, of course, be considered. In our work on tuberculosis, more than 80 rabbits have been injected with BCG but not with red blood cells and their sera tested for isoantibodies. No isoantibodies have been found in these 80 rabbits.

The influence of the BCG inoculation on the specificity of antibody produced in the rabbits after stimulation by red blood cells was tested by comparing specificities of sera produced by animals which received BCG pretreatment plus red blood cells with the specificities of sera produced by animals which received no pretreatment. Seven specific antigens of the rabbit erythrocytes have been studied, and we have found that animals produce specific antibodies in response to the stimulation of known antigenic configurations without reference to pretreatment with BCG.

Through the use of this pretreatment with BCG we have greatly increased the probability of obtaining isoantibodies. The increased number of recipients producing antibody has facilitated the study of the inheritance and immunologic characteristics of isoantigens and their specific antibodies.

Summary. Iso-antibody production in the rabbit is greatly enhanced by pretreatment of the recipients with an intradermal injection of BCG. Of 46 animals given the BCG pretreatment 26 produced isohemagglutinin when stimulated with donor red blood cells, whereas only 3 out of 27 produced isohemagglutinin when the BCG pretreatment was omitted. Antisera to 7 different antigens in the rabbit have been prepared by the use of BCG pretreatment.

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